Atypical Mechanisms of Synthesis, Activation and Release of Interleukin-1β from Vascular Smooth Muscle Cells

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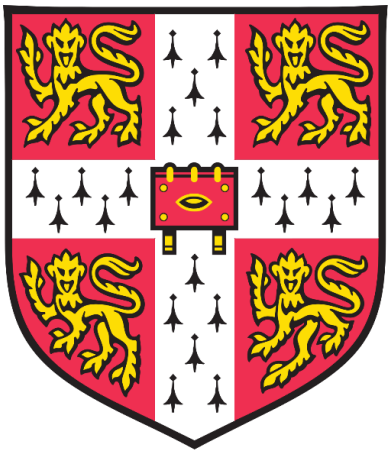

July 2020

This dissertation is submitted for the degree of Doctor of Philosophy

Preface

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

It does not exceed the prescribed word limit for the relevant Degree Committee.

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Abstract

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Atherosclerosis is a widespread disease that often leads to serious conditions such as myocardial infarction and stroke. Chronic inflammation plays an overarching role in its pathophysiology, driving initiation and progression of atherosclerosis in humans and animal models. IL-1β is a potent inflammatory cytokine implicated in atherogenesis and other vascular diseases. However, control of its activation and release has been predominantly studied in macrophages, with scant data on whether vascular smooth muscle cells (VSMCs) can release IL-1β.

I investigated the expression, activation and release of IL-1β from primary human VSMCs. VSMCs expressed caspase-1, -4, and -8 under basal conditions, but not caspase-5. Stimulation of VSMCs with IL-1α and/or LPS caused rapid processing of IL-1β to the mature form, which could be detected by Western and with a mature IL-1β-specific ELISA. Although IL-1β production and cleavage was dependent on NF-κβ activation, neither caspase or NLRP3 inflammasome inhibitors prevented generation of mature IL-1β, suggesting caspase- and NLRP3-independent processing. Instead, production of mature IL-1β was blocked by the serin protease inhibitor dichloroisocoumarin, with cathepsin G the most likely enzyme responsible for pro-IL-1β activation. Interestingly, mature IL-1β was never detected in the conditioned media and instead accumulated to high levels inside VSMCs. VSMCs expressed the pore-forming protein Gasdermin D, and the active form of Gasdermin D permeabilised VSMCs, yet mature IL-1β was still not released. Finally, mature IL-1β extracted from VSMCs has 13-fold lower activity than equal amounts of mature IL-1β from Thp1 cells, however the existence of a binding partner or inhibitor of IL-1β is unlikely the cause of this phenomenon.

In conclusion, the work presented in this thesis suggests that VSMCs have a novel mechanism that specifically prevents the release of active IL-1β, and that perturbation of this could potentially contribute to vascular inflammation and atherogenesis.

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Abbreviations

AIM2	Absent In Melanoma 2
AMPK	AMP-activated protein kinase
ASC	Apoptosis-associated speck-like protein containing a caspase-recruitment domain
ATF2	Activating transcription factor 2
Atg	Autophagy-related
ATP	Adenosine triphosphate
BMP	Bone morphogenetic protein
CCL2	Chemokine (C-C motif) ligand 2
CD	Cluster of differentiation
CLRs	C-type lectin receptors
CM	Conditioned media
CRP	C-reactive protein
CXCL8	C-X-C Motif Chemokine Ligand 8
DAMPs	Damage-associated molecular patterns
DMEM	Dulbecco's Modified Eagle Medium
DNA	Damage-associated molecular patterns
ECACC	The European Collection of Authenticated Cell Cultures
ELISA	Enzyme-linked immunoassay
ER	Endoplasmic reticulum
ERK	Extracellular Receptor Kinase
FCS	Foetal calf serum
FGF2	Fibroblast growth factors 2
HASMCs	Human artery smooth muscle cells
HMGB1	High mobility group box 1
ICAM-1	Intercellular Adhesion Molecule *1*
IETD	Z-IETD-FMK
IFN	Interferon
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1Ra	Interleukin-1 receptor antagonist
IL-1RAcP	Interleukin-1 receptor accessory protein
IP-10	Interferon gamma-induced protein 10
IPAF	Ice protease-activating factor
IRAK	Interleukin-1 receptor–activated protein kinase
IRF	Interferon-regulatory factor
I-TAC	Interferon-inducible T-cell alpha chemoattractant
Iκβ	Nuclear factor kappa-B inhibitor
JNK	C-Jun N-terminal kinase
LEVD	Z-LEVD-FMK
LGP	Laboratory of genetics and physiology
LLME	Leu-Leu-O-methyl ester
LPS	Lipopolysaccharides
LRRs	Leucine-rich repeats
MAPK	Mitogen-activated kinase
MCP	monocyte chemoattractant protein
MDA	Melanoma differentiation associated factor
MKK	MAP kinase kinase
MLKL	Mixed lineage kinase domain-like
MMP	Matrix metalloproteinase
MSU	Monosodium urate
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response gene 88
NAIP	Neuronal Apoptosis Inhibitory Protein

NF-κβ	Nuclear factor-kappa B
NLR	NOD-like receptor
NLRP	NOD-like receptor pyrin-containing
NOD	Nucleotide oligomerization domain
NP-40	Nonidet P-40
pAb	Polyclonal Antibody
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PR3	Proteinase 3
PRRs	Pattern recognition receptors
PSMCs	Pulmonary smooth muscle cells
QVD	Q-VD-OPH
RAGE	Receptor for advanced glycation end-products
RIG-I	Retinoic acid-inducible gene I
RLRs	RIG-I like receptors
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
SAA	Serum amyloid A
SCI	Systemic chronic inflammation
SEM	Standard error of the mean
Serpin B9	Serpin proteinase inhibitor 9
SiRNA	Small interfering RNA
SMCs	Smooth muscle cells
SYK	Spleen tyrosin kinase
TAB	TGF-Beta Activated Kinase 1 (MAP3K7) Binding Protein
TAK	TGF-β-activated kinase
TGF	Transforming growth factor
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	Tumour necrosis factor–associated factor
VCAM	Vascular cell adhesion molecule
VSMCs	Vascular Smooth muscle cells
YVAD	Z-YVAD-FMK
ZAAD	Z-AAD-CH2Cl
ZVAD	Z-VAD-FMK

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1. Introduction

1.1 Overview of the immune system

Vertebrate animals are surrounded by and in constant interaction with beneficial and pathogenic microorganisms[1]. Hence, these species have acquired defence mechanisms to preserve homeostasis and develop immunity[1]. The term "immunity", derives from the Latin word *immunitas,* meaning exempt, and was adopted to refer to resistance against infection[2].

In mammals, the immune system is an interconnected network composed of cells and molecules that in physiological conditions recognise and eliminate pathogens and noxious proteins, avoiding excessive destruction of host tissue or removal of commensal microorganisms[1, 3]. The immune system possesses two levels of defence to carry out its functions: innate and adaptive immunity[2].

1.1.1 Innate immunity

Innate immunity is the frontline response to pathogens and is characterised by being highly effective, immediate (acting within minutes to hours after exposure), but nonspecific[2, 4]. It comprises physical, chemical and cellular barriers including: skin, mucous membranes and secretions that prevent entry of potential invaders into the organisms; phagocytic cells (e.g. neutrophils, monocytes, macrophages) that engulf and destroy pathogens and foreign particles; peptides and proteins with antimicrobial activity (e.g. defensins, cathelicidins, histatins, phospholipase A2, lysozyme, complement); cellular receptors (e.g. pattern recognition receptors) that identify pathogen-associated molecular patterns (PAMPs) expressed by microorganisms, or damage-associated molecular patterns (DAMPs) typically released from damaged cells; as well as cytokines and other mediators that orchestrate inflammation[2, 4-6].

1.1.1.1 Pattern recognition receptors

Pattern recognition receptors (PRRs) are proteins expressed mainly by immune cells that sense molecular motifs on pathogens and damaged tissues, leading to cellular activation and production of cytokines, chemokines and other proinflammatory proteins[2, 5]. The existence of these receptors was initially proposed by Janeway in 1989[7], and confirmed when the Toll-like receptors (TLRs) were identified years later[8]. Since then, multiple receptors have been characterised and classified into several families:

Toll-like receptors: TLRs are transmembrane proteins composed of N-terminal leucine-rich repeats (LRRs) and a cytoplasmic Toll/IL-1R homology (TIR) domain[3]. During infection, molecular components of pathogens bind to LRRs and cause the activation of the TIR domain, which recruits cytoplasmic adaptor proteins (e.g. MyD88 or MAL) that initiate signalling cascades, and ultimately lead to NF-κβ and IRF induction[3].

Up to now 10 TLRs have been described in humans that sense different ligands. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are located in the plasma membrane and recognise bacteria, viruses, fungi and parasites[9]. In contrast, TLR3, TLR8, TLR9 and TLR10 are found in endolysosomal membranes and identify nucleic acids (RNA and DNA) expressed by ingested or intracellular bacteria and viruses[9].

C-type Lectin receptors: C-type lectin receptors (CLRs) are transmembrane proteins synthesised mainly by immune cells (e.g. neutrophils, monocytes, macrophages, dendritic cells) that sense carbohydrates present in microorganisms or endogenous molecules[10]. The CLRs facilitate the internalisation of antigens and promote the synthesis of inflammatory mediators through activation of NF-κβ, type I interferon and inflammasome signalling pathways[10].

RIG-I like receptors: RIG-I like receptors (RLRs) are a family of cytosolic helicases that recognise viral RNA and trigger the expression of interferon and interferon-stimulated genes[11]. Retinoic acid-inducible gene I (RIG-I), melanoma differentiation associated factor 5 (MDA5), laboratory of genetics and physiology 2 (LGP2) are members of the RLRs family and are found in multiple tissues[11].

NOD-like receptors: Nucleotide-binding and oligomerization domain-like receptors (NLRs) are cytoplasmic proteins formed by C-terminal LRRs, a central NOD domain and an N-terminal effector domain[12]. NLRs play an important role in immunity by detecting intracellular PAMPs and DAMPs, which leads to the subsequent activation of the inflammatory response via the NF-κβ pathway or inflammasome formation[12] (see below).

22 NLRs have been described in humans, which can be classified into 4 subfamilies according to the N-terminal domain: NLRA, NLRB, NLRC, and NLRP[12]. Likewise, previous investigations found that vascular smooth muscle cells (VSMCs) express diverse PRRs including TLRs (e.g. TLR3, TLR4, TLR6)[13, 14], NLRs (e.g. NOD2, NLRP3, NLRC4)[15, 16] and RLRs (e.g. RIG-I in response to interferon gamma)[17].

1.1.1.2 Inflammasomes

Inflammasomes are intracellular protein complexes that assemble in response to PAMPs and DAMPs that regulate the activation of caspase-1, a protease that subsequently processes the cytokines pro-IL-1β and pro-IL-18[2].

The components of the inflammasome include a sensor protein, the adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and the enzyme caspase-1[18, 19]. Several inflammasomes have been identified and the best characterised are NLRP3, NLRP1, IPAF and AIM2 (Fig. 1).

The NLRP1 inflammasome was the first to be described[2]. It is present in neurons and immune cells (e.g. neutrophils, monocytes, dendritic cells, B and T cells)[2]. NLR pyrin-containing 1 (NLRP1) is the sensor that detects insults including the *Bacillus anthracis* toxin; in fact, the isoform Nlrp1b has been associated with an increased risk of anthrax infection[2]. Interestingly, NLRP1 is activated by K+ efflux and activates pro-caspase-1 and pro-caspase-5[20].

However, the most extensively studied is the NLRP3 inflammasome that contains NOD-like receptor pyrin-containing 3 (NLRP3) as the sensor[18]. The inducers and process for activation of the NLRP3 inflammasome is described in detail below.

Another important inflammasome is IPAF, which is composed of a Neuronal apoptosis inhibitory protein (NAIP) as the receptor that recognises flagellin (NAIP5 and NAIP6), needle (NAIP1) and rod (NAIP2) proteins of the Gram-negative bacteria type III or IV secretion systems (e.g. *Salmonella typhimurium, Shigella flexneri, Legionella pneumophila,* and *Pseudomonas aeruginosa)*[20, 21]. Subsequently, NAIP interacts with NLR containing a caspase activating and recruitment domain 4 (NLRC4) and activates pro-caspase-1[21]. The IPAF inflammasome is involved in other cellular processes such as autophagy, T cell activation, and production of antibodies[21].

Finally, the AIM2 inflammasome contains the protein AIM2 that senses foreign or self-double-stranded DNA[20], and is important in the recognition of bacteria (e.g. *Francisella tularensis, Listeria monocytogenes, Streptococcus pneumonia, Staphylococcus aureus*) and viruses (e.g. cytomegalovirus, Epstein-Barr virus, Herpes-simplex virus type 1)[22]. The AIM2 inflammasome has been associated with the development of autoimmune diseases and cancer[22].

Interestingly, previous studies demonstrated that components of the NLRP1, NLRP3 and NLRC4 inflammasomes are expressed by control VSMCs and overexpressed in senescent VSMCs[16]. Likewise, AIM2 is also present in VSMCs derived from normal arteries and atherosclerotic plaques[23, 24].

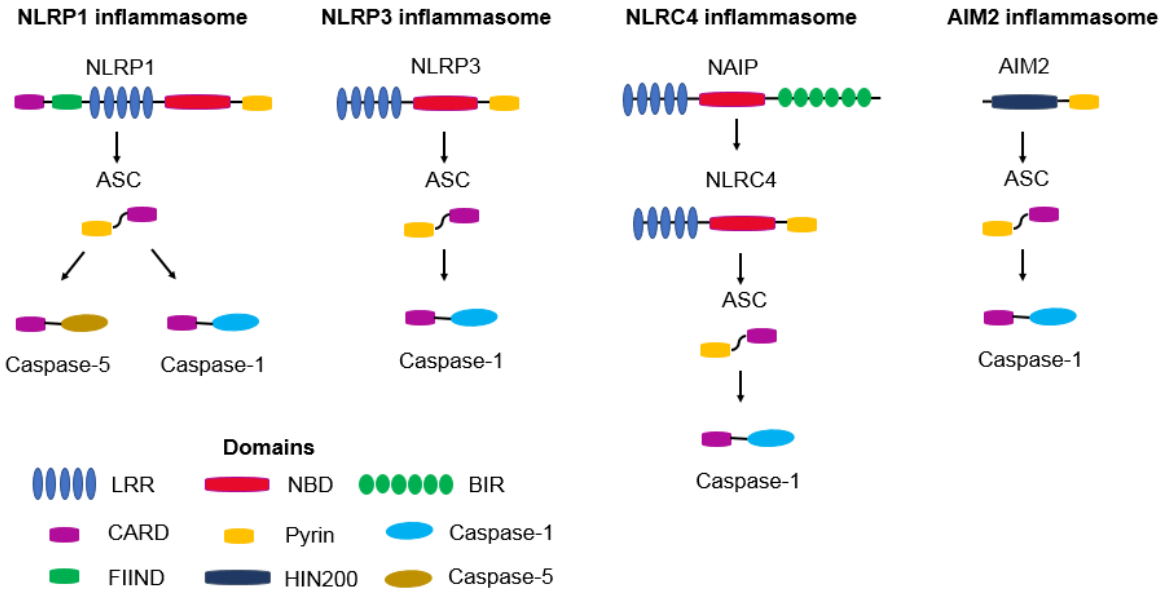


Fig. 1. Structure of the best studied inflammasomes: NLRP1, NLRP3, NLRC4 and AIM2 inflammasomes are macromolecular platforms that contain a sensor, an adaptor (ASC) and pro-caspase-1 or-5 (NLRP1), with their assembly and activation inducing processing of IL-1β and IL-18. Adapted from *Hornung et al., 2010.*

1.1.2 Adaptive immunity

Adaptive immunity (also called acquired immunity) is the second line of defence against infections. It is more complex and takes longer to develop than innate (usually between 4 – 5 days) and encompasses responses tailored to destroy a specific pathogen[3]. A cardinal feature of adaptive immunity is the ability to create immunological memory that allows quicker detection and neutralisation of invaders after a second exposure to them[3].

The cells that mediate adaptive immunity responses are CD8+ T lymphocytes that eliminate infected cells, CD4+ T lymphocytes that activate and regulate other immune cells through secretion of cytokines and B cells that are responsible for the secretion of antibodies[3].

1.2 Inflammation

The term “inflammation” derives from the Latin *iflammatio*, which means ignite or set fire[25] and is defined as the physiological response to infection or injury[5]. The clinical manifestations of the inflammatory process were initially described by the doctor Aulus Cornelius Celsus in the first century AD, and are recognised as the "four cardinal signs of inflammation": *rubor* (redness), *calor* (increased heat), *tumor* (swelling) and *dolor* (pain). Later, Rudolph Virchow introduced *functio laesa* (loss of function) as the fifth sign[26].

Classically, the inflammatory pathway is composed of inducers, which trigger the inflammatory response; sensors, responsible for the recognition of the inducers; mediators, which are activated by the sensors; and finally, the target tissues[27]. Distinct inflammatory pathways have been described and are activated in response to different types of stimuli (Fig. 2)[27].

During bacterial infections, the invasion of epithelial barriers by pathogens leads to their recognition by PPRs (e.g. TLRs) on tissue-resident macrophages, which then synthesise and secrete cytokines (e. g. IL-1, IL-6, TNF), chemokines (e. g. CCL2, CXCL8), prostaglandins and leukotrienes[27, 28]. These mediators of inflammation cause diverse effects on local tissues including vasodilation, increased vascular permeability and extravasation of protein-rich plasma, neutrophils, macrophages and mast cells in order to eliminate the threat[27, 28]. Similarly, systemic effects such as fever, anorexia, fatigue, as well as production of C-reactive protein and coagulation factors may occur in response to IL-1, IL-6 and TNF[27]. At the same time, bacteria are recognised by receptors on tissue-resident dendritic cells, which initiate the adaptive immune response[6]. The role of dendritic cells, along with macrophages and B cells, is to engulf and destroy pathogens and subsequently present their antigens to T lymphocytes localised in peripheral lymphoid organs[6]. The antigen-presenting cells also secrete cytokines, including IL-1, that modulate the innate and adaptive response[6].

Additional cellular and humoral mechanisms mediate the inflammatory response to viral and parasitic infections. For example, the generation of type-I interferons (IFN-α, IFN-β) is triggered by virus-infected cells, and CD8+ T lymphocytes are also activated in order to destroy the pathogen[27]. Likewise, intracellular parasites lead to CD4+ and CD8+ T lymphocyte activation and IFN-γ synthesis, while extracellular parasites (e.g. nematoda and platyhelminthes) stimulate CD4+ T lymphocytes, eosinophils, mast cells and basophils, as well as production of histamine, IL-3, IL-4, IL-5, IL-9, IL-10 and IL-13[2, 27].

Furthermore, endogenous signals released by damaged tissues (DAMPs) (e.g. nuclear proteins, mitochondrial DNA, components of extracellular matrix and cellular chaperones) can

also trigger sterile inflammation[27, 29]. These molecules are sensed by PPRs on tissue-macrophage and again initiate the inflammatory cascade[27].

Finally, the resolution of inflammation usually occurs after elimination of the threat and restoration of the damaged tissues[27]. This process is mediated by anti-inflammatory prostaglandins and lipoxins that inhibit the recruitment of neutrophils and promote the migration of monocytes to the site of injury in order to remove cell debris and promote tissue healing[27]. Nevertheless, inflammation can persist under certain circumstances such as chronic infections, exposure to allergens, endogenous crystals and indigestible foreign materials[27].

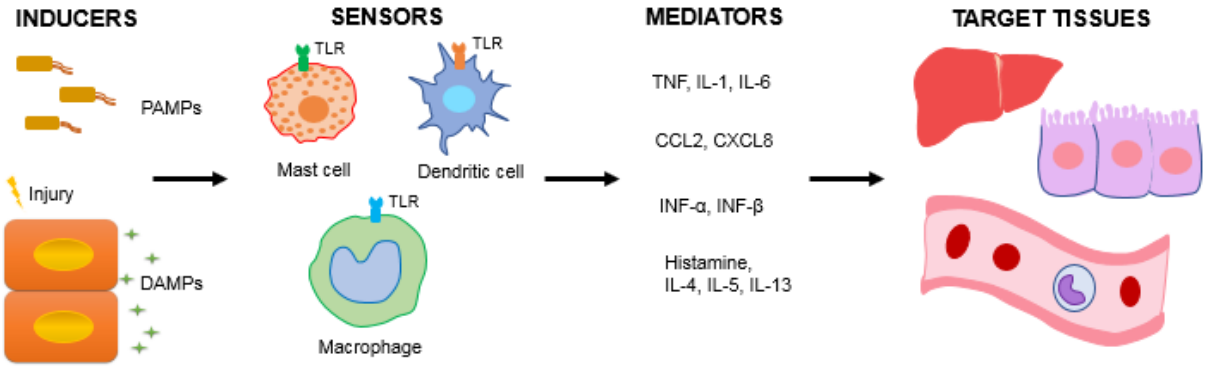


Fig. 2. The components of the inflammatory pathway: PAMPs and DAMPs (inducers) are detected by receptors (sensors) and initiate signalling pathways that lead to the synthesis and secretion of molecules (mediators) which can produce diverse effects on distinct organs (target tissues). Adapted from *Medzhitov, 2010.*

1.2.1 Role of inflammation in health and disease

The inflammatory response is essential to maintain homeostasis by allowing the identification, neutralisation and elimination of insults, while stimulating tissue repair[30]. Therefore, multiple diseases originate as a consequence of defects in innate or acquired immunity and are called primary and secondary immunodeficiency, respectively[31]. The clinical picture of immunodeficiency varies depending on the immune system alteration and includes predisposition to infections and malignancies, autoimmune manifestations, granulomatous disorders and atopy, amongst others[31].

However, numerous other pathologies are produced by intense or prolonged inflammation[27]. The local effects of chronic inflammation are diverse and include formation of granulomas, development of tertiary lymphoid structures and tissue remodelling[27]. Similarly, systemic

chronic inflammation (SCI) plays an important role in the pathogenesis of highly prevalent chronic diseases[32].

SCI is a long-term low-grade inflammation usually caused by DAMPs and linked to increased risk of type-2 diabetes, dyslipidaemia, chronic kidney disease, malignancies, depression, osteoporosis, neurodegenerative pathologies, autoimmune disorders and cardiovascular diseases[32].

1.2.1.1 Atherosclerosis and inflammation

Atherosclerosis is a chronic disease implicated in the pathogenesis of other cardiovascular pathologies, and is the primary cause of mortality in the world[30, 33]. It is characterised by accumulation of lipids, infiltration of immune cells, proliferation of smooth muscle cells and deposition of connective tissue in the intima of large and medium-sized arteries[34, 35].

The prevailing views about the genesis and development of atherosclerosis has changed over the last few decades. 30 years ago atherosclerosis was considered only a “lipid storage disease” that caused complications due to obstruction of inanimate conduits[36]. At the present time the endothelium is well known to be an active ‘tissue’, and inflammation is accepted as the fundamental process that drives atherosclerotic plaque formation (atherogenesis). This is based on data collected from numerous pieces of experimental research, epidemiological studies and clinical trials[36-38].

The process of atherogenesis is progressive and although it can start in the prenatal period, typical lesions establish over 20 – 30 years with no clinical manifestations[34]. The stages of atherogenesis have been elucidated based on experimental studies in animals and clinical investigations, which integrate to form a model that has widespread acceptance in the scientific community - the “response to injury” hypothesis[30] (Fig. 3).

According to this hypothesis, the initial step in atherosclerosis is dysfunction and injury of the endothelial cells, as a consequence of chemical and physical insults such as hyperlipidemia, dysglycemia, hyperhomocysteinemia, immune complex deposition, hemodynamic shear stress and irradiation[30].

The damage to endothelial cells is followed by alteration of their homeostatic functions, including anticoagulation and selective permeability[39]. As a result, leukocytes (e.g. monocytes, dendritic cells, T lymphocytes and mast cells) adhere to the endothelium and migrate through cellular junctions, or by transcytosis, to the intima[35]. Expression of leukocyte adhesion

molecules on the surface of endothelial cells (e.g. vascular cell adhesion molecule 1, VCAM-1; intercellular adhesion molecule 1, ICAM-1; E-selectin; P-selectin), along with the expression of chemokines (e.g. monocyte chemoattractant protein 1, MCP-1; IP-10, I-TAC) by endothelial and smooth muscle cells, drive recruitment of leukocytes into the arterial wall[35].

Once monocytes are in the intima they differentiate into macrophages and start to imbibe lipids, becoming foam cells or lipid-laden macrophages. This lipid internalization is mediated by scavenger receptors, such as CD36, macrosialin and others [35]. This accumulation of foam cells in the arterial wall leads to the formation of fatty streaks, which are precursor lesions of atheromas without clinical significance and with the potential to regress[35].

However, as atherogenesis progresses, VSMCs begin to play a pivotal role in the next stages of disease. VSMCs migrate into the intima, multiply and adopt a dedifferentiated secretory phenotype, characterised by increased synthesis and release of extracellular matrix (e.g. collagen, elastin and proteoglycans) that contributes to the formation of a fibrous cap[35]. Matrix components, such as proteoglycans and glycosaminoglycans, attract and retain lipids in the plaque, the lipids are oxidised and this increases the recruitment of macrophages to the lesion[40]. Likewise, some VSMCs uptake lipoproteins and transdifferentiate into macrophages and foam cells. Osteochondrogenic and mesenchymal stem cell-like cells have also been identified in atherosclerotic plaques[40].

Later, apoptosis of VSMCs leads to plaque destabilisation[41], and the inflammatory response is amplified by secondary necrosis of uncleared apoptotic cells derived from inefficient efferocytosis[40]. Interestingly, another phenomenon that contributes to plaque instability and inflammation is the senescence of VSMCs[40]. Senescent VSMCs proliferate in a lower rate compared to normal VSMCs, synthesise less extracellular matrix components and develop a senescence-associated secretory phenotype driven by IL-1α which is characterised by release of high concentrations of proinflammatory cytokines, chemokines and metalloproteinases[16]. Finally, the rupture or erosion of the atherosclerotic plaque triggers thrombosis and lead to vessel occlusion - the main events in the pathophysiology of myocardial infarction and stroke[35, 39, 40].

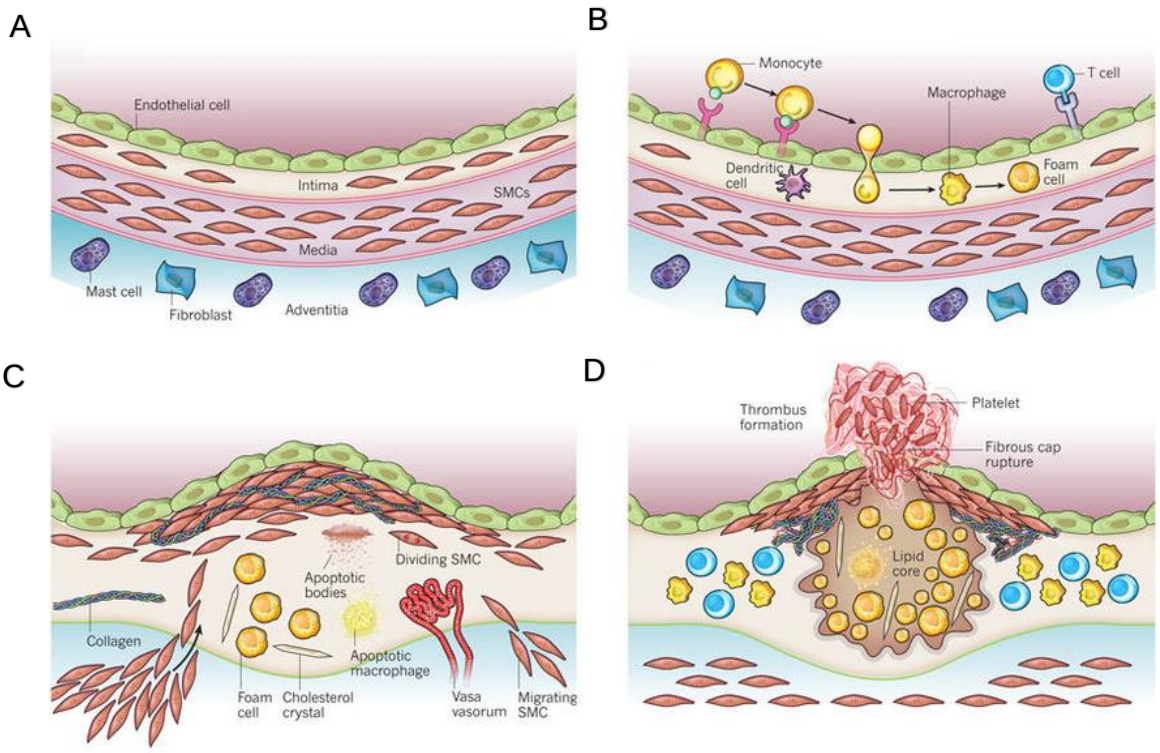


Fig. 3. Phases of atherosclerotic plaque development: structure of a normal artery which is composed of three layers: the tunica intima, media and adventitia **(A).** Initially, leukocytes adhere to the endothelium and migrate into the tunica intima because of endothelial damage. Monocytes in the tunica intima turn into macrophages and imbibe lipids (foam cells) **(B)**. Later, VSMCs migrate from the tunica media to the tunica intima where they proliferate and synthesise extracellular matrix. In advanced lesions, leukocytes and vascular smooth muscle cells can undergo cell death, which forms the necrotic or lipid core **(C)**. Finally, rupture of the plaque triggers activation of coagulation and thrombosis, which drives the clinical consequences of atherosclerosis. Adapted from *Libby et al., 2011.*

Finally, new inflammatory biomarkers have been described as risk predictors of cardiovascular disease (e.g. C-reactive protein, IL-6, IL-18, TNF-α) that are independent of traditional risk factors[38]. Therefore, investigating inflammation and its impact on plaque biology is essential, because it represents a promising therapeutic target to prevent and treat cardiovascular diseases[38].

1.3. Interleukin-1 family

The IL-1 family is a set of cytokines that comprises 7 agonistic ligands (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ), 3 antagonistic ligands (IL-1Ra, IL-36Ra, IL-38), 1 anti-inflammatory molecule (IL-37) and 10 receptors (Table 1)[42, 43]. IL-1α, IL-1β, IL-1Ra, IL-18, IL-33 and IL-36Ra are all composed of 12 anti-parallel β-strands that form the β-trefoil structure characteristic of the family[44].

Table 1. Members of IL-1 family, adapted from *Garlanda et al., 2013.*

Cytokine	Receptor	Coreceptor	Activity
IL-1α	IL-1R1, IL-1R2	IL-1RAcP	Alarmin, inflammation, Th17 cell responses.
IL-1β	IL-1R1, IL-1R2	IL-1RAcP	Antimicrobial resistance, inflammation, Th17 cell responses.
IL-1Ra	IL-1R1		Inhibition of inflammation.
IL-18	IL-18Rα	IL-18Rβ	Inflammation, Th1 cell responses.
IL-33	ST2	IL-1RAcP	Inflammation, Th2 cell responses.
IL-36α	IL-36R	IL-1RAcP	Skin and lung inflammation.
IL-36β	IL-36R	IL-1RAcP	Skin and lung inflammation.
IL-36γ	IL-36R	IL-1RAcP	Skin and lung inflammation.
IL-36Ra	IL-36R		Inhibition of inflammation.
IL-37	IL-18Rα		Inhibition of inflammation.
IL-38	IL-36R		Inhibition of inflammation.
	SIGIRR		Inhibition of inflammation.
	IL1RAPL2		Not known.
	TIGIRR-2		Not known.

IL-1α and IL-1β are the most studied ligands and their biological activities are similar due to the fact they both signal through the same receptor: the type 1 IL-1 receptor (IL-1R1).

1.3.1 Role of IL-1 in innate and adaptive immunity

Concerning the role of IL-1 in innate immunity, IL-1R1 and TLRs possess a highly homologous cytoplasmic domain (TIR); therefore, the proinflammatory responses to IL-1 and TLR ligands, such as synthesis of cyclooxygenase type 2, type 2 phospholipase A, inducible nitric oxide synthase, are practically identical[45, 46].

IL-1 also stimulates the production of adhesion molecules and chemokines that promote extravasation of immune cells to the site of injury[46]. Similarly, IL-1 induces differentiation of

myeloid stem cells, and the synthesis of acute-phase proteins (e.g. CRP, SAA) and other cytokines (e.g. IL-6)[46]. In addition, IL-1 contributes to the adaptive immune response through costimulation of T cells, promotion of thymocyte growth, stimulation of Th2 polarization and Th17 activity. IL-1 also stimulates B cell expansion and antibody generation[46].

1.3.2 IL-1 signalling pathway

The IL-1 signalling pathway has been well characterised. In inflammation, DAMPs or PAMPS are detected by PRRs on immune cells, which triggers production of pro-IL-1α and pro-IL-1β[47]. Later, these cytokines bind to the IL-1R1, followed by recruitment of the IL-1R accessory protein (IL-1RAcP) to form a trimeric complex that subsequently recruits myeloid differentiation primary response gene 88 (MyD88) and interleukin-1 receptor–activated protein kinase (IRAK)4[48, 49]. IRAK4 is activated after autophosphorylation, and subsequently phosphorylates IRAK1 and IRAK2, allowing oligomerization of tumour necrosis factor–associated factor (TRAF) 6[49], which binds to TGF-β-activated kinase 1 (TAK1) and TAK1-binding proteins TAB1 and TAB2 localised in the plasma membrane[50]. Subsequently, the TAK1-TAB1-TAB2-TRAF6 complex returns to the cytosol where ubiquitination of TRAF6 and phosphorylation of TAK1 takes place[50].

After this, two different pathways can be followed[50]. In the first pathway, interaction of TAK1 with MAP kinase kinase (MKK) activates mitogen-activated kinase (MAPK) p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), leading to the activation of diverse transcription factors including c-Jun, c-Fos, c-Myc and ATF2[50]. These transcription factors regulate diverse proteins that promote proliferation and migration of VSMCs, upregulation of metalloproteinases and induction of foam cell formation[51-55].

In the second pathway, TAK1 can active the inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ), which leads to phosphorylation and subsequent degradation of the nuclear factor kappa-B inhibitor (Iκβ) and activation of NF-κβ[50]. NF-κβ controls transcription of proinflammatory genes and the activation of endothelial cells and macrophages, proliferation of VSMCs, expression of adhesion molecules and increased vascular permeability - all key events in atherogenesis (Fig. 4)[49, 56].

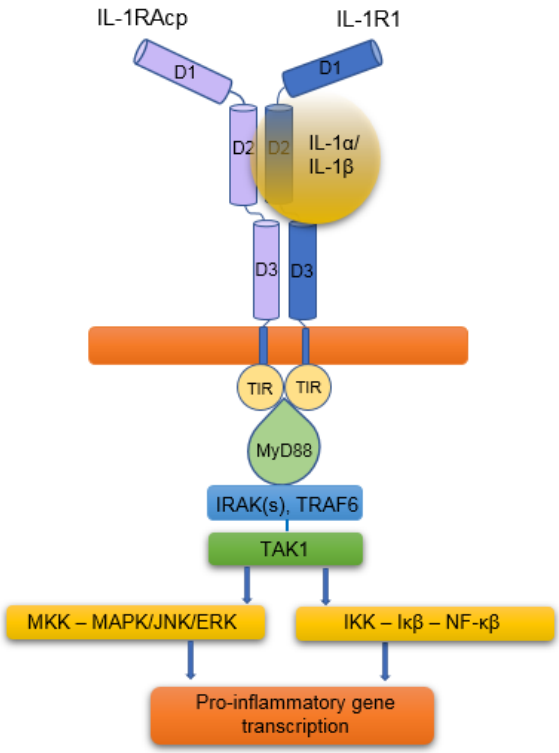


Fig. 4. The IL-1 signalling pathway: IL-1 binds to IL-1R1 and recruits IL-1RAcp to form a complex that triggers signalling cascades to induce transcription of pro-inflammatory genes [48, 49]. D1, D2, D3 = ectodomains of IL-1 family receptors, TIR = intracellular Toll/IL-1R domains. Adapted from *Deng, 2014.*

The activation of the IL-1 signalling pathway can be blocked by IL-1RA, a protein that competitively inhibits the binding of IL-1α and IL-1β to the IL-1R1 (Fig. 5A)[44]. Similarly, IL-1R2, a decoy receptor, binds to IL-1α or IL-1β and can form a trimeric complex with IL-1RAcP, however the lack of a TIR domain in IL-1R2 prevents IL-1 signal transduction (Fig. 5B)[44, 45].

Although they signal through the same receptor, some differences between IL-1α and IL-1β have been identified: firstly, they are encoded by different genes and the regulation of expression in response to stimuli is different[57]; secondly, IL-1α is constitutively expressed in cells that compose the epithelium of the gastrointestinal tract, lung, kidney, skin, endothelium and astrocytes, whereas IL-1β is synthesised mainly by myeloid cells of the monocytic lineage, and brain microglia; thirdly, IL-1α can exist as a form attached to the membrane of cells, thus exerting local effects, whereas IL-1β is released from cells and can act locally and systemically[43, 57].

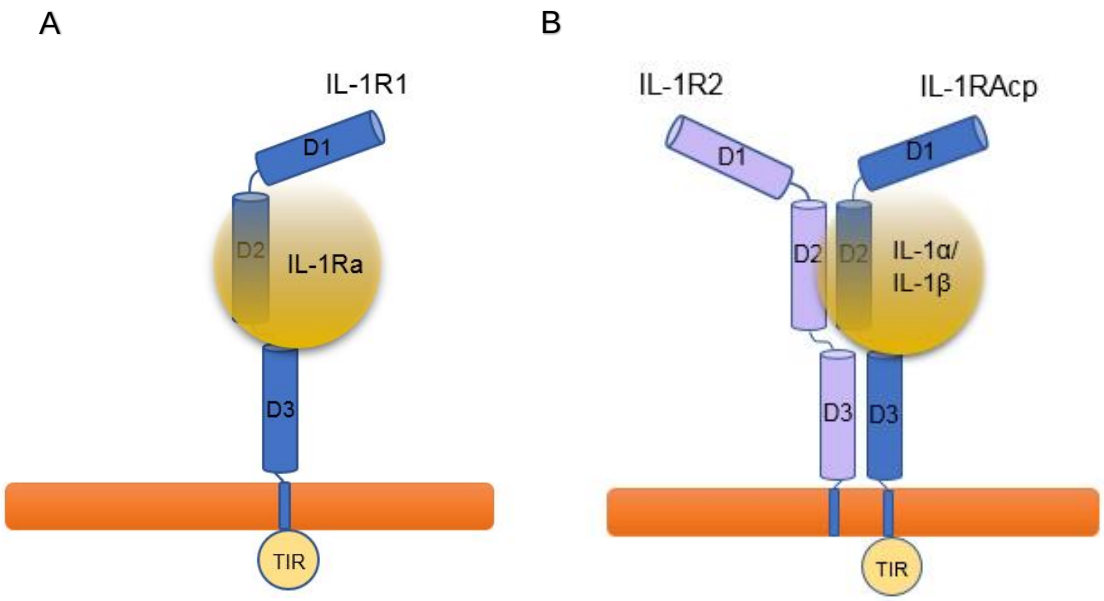


Fig. 5. Antagonism of IL-1 signalling pathway, IL-1Ra, a competitive inhibitor of IL-1 **(A),** IL-1R2, a decoy receptor for IL-1 **(B)**. D1, D2, D3 = ectodomains of IL-1 family receptors, TIR = intracellular Toll/IL-1R domains. Adapted from *Deng, 2014.*

1.3.3 IL-1β and cardiovascular diseases

IL-1β has been associated with diverse conditions due to these extensive and systemic effects, including rare autoinflammatory disorders (e.g. familial cold autoinflammatory syndrome, Muckle-Wells syndrome and chronic infantile neurological cutaneous and articular syndrome), sepsis, and other more prevalent illnesses such as metabolic, autoimmune, neurodegenerative and cardiovascular diseases[57-59].

The role of IL-1 in cardiovascular diseases is not completely clear. Numerous experimental studies have established the pro-atherogenic role of IL-1[60-70] and IL-1α$^{-/-}$ mice exhibit reduced atherosclerotic lesions[65, 68, 70], however IL-1β$^{-/-}$ mice developed lesions that are either smaller or no different[65, 70]. Likewise, although smaller plaques were found in IL-1R1$^{-/-}$ mice, they were more unstable - suggesting that IL-1 might promote stabilization of plaques[71]. Also, although the induction of inflammasomes by cholesterol crystals triggers activation of IL-1β[67, 72], lack of the inflammasome components NLPR3 or ASC led to formation of smaller plaques[67] or had no effect[73]. In contrast, the results obtained from clinical studies show increased IL-1β in atherosclerotic vessels compared to normal vessels[74], and a positive correlation exists between risk factors for coronary disease and serum levels of IL-1β[75-77].

Furthermore, the results published recently of the large clinical trial Canakinumab AntiNflammatory Thrombosis Outcomes Study (CANTOS) showed that the use of a human monoclonal antibody against IL-1β reduces the recurrence of cardiovascular events in patients who had suffered a previous myocardial infarction and had high levels of C-reactive protein as well as low lipid levels, confirming the association of this cytokine and atherosclerotic disease[78].

1.3.4 Synthesis and activation of IL-1β

As mentioned above, IL-1β is primarily produced by brain microglia and cells of the monocytic lineage (e.g. monocytes, macrophages and dendritic cells). However, it is also expressed by vascular smooth muscle cells treated with tumour necrosis factor, bacterial endotoxin or recombinant IL-1α and IL-1β[43, 79, 80]. But, our knowledge about its processing, activation and release is derived almost exclusively from observations made on immune cells.

According to this data, when these immune cells are in contact with stimuli such as DAMPs or PAMPs (e.g. lipopolysaccharide) that are recognized by PRRs (e.g. TLRs), this induces expression of pro-IL-1α/β and inflammasome components such as NLRP3 – this is known as the priming step[18, 47, 81] .

Later, a second stimulus (additional PAMPs or DAMPs; e.g. ATP) that generates cellular potassium efflux, oxidative stress or lysosome rupture, is sensed by NLRP3 and leads to recruitment of ASC and formation of the NLRP3 inflammasome that triggers caspase-1 autoactivation[19]. Finally, caspase-1 processes the biologically inactive pro-IL-1β to its mature form. This is known as the canonical inflammasome activation pathway [18] (Fig. 6).

However, other non-canonical mechanisms of inflammasome activation have been established[82] - Fig. 6. On immune cells, PAMPs sensed by TLRs or IFN signalling lead to expression of caspase-11 (mice) or caspase-4/5 (humans), and stimulation of pro-IL-1β expression. Subsequently, cytosolic LPS from intracellular Gram negative bacteria, LPS endocytosed by CD14 or LPS escaping from endocytosed bacteria forms a complex with caspase-11, -4, or -5 leading to their autoactivation, caspase cleavage of Gasdermin D to the active N-terminal form that binds to the plasma membrane and induces pore formation, and subsequent release of IL-1β. At the same time, the active Gasdermin D N-terminal triggers formation of canonical NLRP3 inflammasomes - see below-[82-85].

Moreover, recent studies indicate that caspase-8 can also play a role in IL-1β processing by different mechanisms. Firstly, after TLR3 or TLR4 ligation caspase-8 can directly cleave IL-1β

at the same site that caspase-1 targets (Asp 116)[86, 87]. Secondly, in immune cells after certain fungal and mycobacterial infections, dectin 1 and the adaptor SYK causes assembly of a CARD9–BCL-10–MALT1 complex that promotes IL-1β transcription. This complex also binds to ASC and caspase-8 leading to maturation of pro-IL-1β[87]. Finally, caspase-8 can activate caspase-1 in response to canonical inflammasome activators[87].

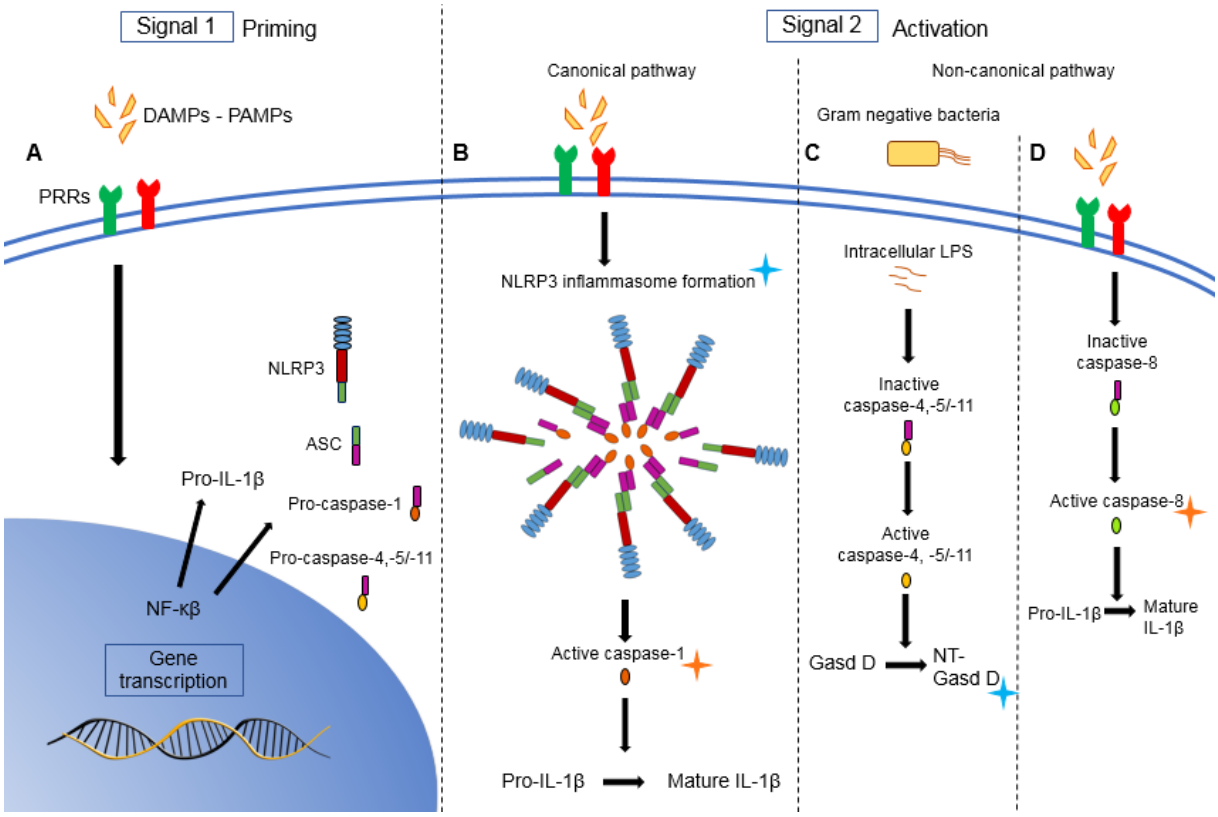


Fig. 6. IL-1β activation in macrophages is mediated by canonical and non-canonical inflammasomes, DAMPs and/or PAMPs triggers the gene expression of pro-IL-1β and components of the NLRP3 inflammasome or caspase-4,-5/-11 **(A)**. Later, a second stimulus produces the formation of the NLRP3 inflammasome that activates caspase-1, which in turn cleaves pro-IL-1β into mature IL-1β **(B)**. Alternatively, intracellular LPS leads to the activation of caspase-4/5 or -11, which processes gasdermin D into NT-Gasdermin D. Subsequently, NT-Gasdermin D induces pyroptosis and NLRP3 inflammasome assembly **(C)**. Moreover, diverse stimuli can activate caspase-8 that can mediate IL-1β cleavage directly, or through activation of caspase-1 **(D)**. Adapted from *Rader, 2011.*

Interestingly, the cleavage of IL-1β can be also mediated by other proteases. Studies performed in mice deficient in caspase-1 demonstrated that proteinase 3 (PR3) is implicated in the activation of IL-1β in acute neutrophil-dominated arthritis[88]. In addition, the release of IL-1β from Thp1 cells increased in response to incubation with purified PR3 and coculture with neutrophils[89]. Likewise, neutrophil elastase, cathepsin G, mast cell chymase, matrix metalloproteinases and granzyme A can process pro-IL-1β[90-93]

1.3.5 Secretion of IL-1β

The machinery and pathways involved in the synthesis and activation of IL-1β are well established in immune cells. However, the mechanisms of IL-1β release are controversial and not completely understood[47].

We know that IL-1β lacks a leader sequence, and thus it is not secreted after translocation into the endoplasmic reticulum, followed by transportation from Golgi apparatus to the cellular membrane and extracellular milieu[94, 95]. Thus, several hypotheses have been raised to explain the unconventional pathways of IL-1β secretion (Fig. 7).

One initial hypothesis proposed that lysis of cells was required to produce IL-1β externalization[94]. Later, however, investigations showed that living human monocytes activated by LPS can secrete mature IL-1β independent of the classical ER-Golgi route[96]. These conclusions were based on the presence of mature IL-1β and absence of pro-IL-1β in the conditioned media of activated monocytes, the lack of mature IL-1β and an abundance of pro-IL-1β inside monocytes, and the failure to block IL-1β release after treatment with drugs that inhibit the classical secretory pathway (e.g. Brefeldin A, Monensin)[96].

An alternative route proposed to explain the release of IL-1β is exocytosis by lysosomes. In monocytes stimulated with LPS, investigations demonstrated that pro-IL-1β and caspase-1 can be found inside of lysosomes, and external activators such as ATP trigger increased intracellular calcium that promotes the mobilization of lysosomes along microtubules. Finally, these vesicles dock and fuse to the cellular membrane allowing the exit of IL-1β[96, 97]. These studies also suggested that IL-1β is processed by caspase-1 inside of the lysosomes before or immediately after exocytosis[98].

Moreover, extracellular ATP detected by the $P2X_7$ receptor on the surface of Thp1 cells, microglia, astrocytes, neutrophils, and dendritic cells can induce the formation of the NLRP3 inflammasome, by a process mediated by potassium efflux[99-105]. This complex triggers maturation of IL-1β by caspase-1 and translocation of phosphatidylserine from the inner to the outer leaflet of the cellular membrane leading to subsequent release of active IL-1β-containing microvesicles, with sizes varying from 100nm to 1μm[97 100, 102, 106]. Other stimuli that induce NLRP3 assemble and subsequent release of IL-1β have been characterised, and include nigericin, maitotoxin, *Staphylococcus aureus* infection, monosodium urate crystals, calcium pyrophosphate dihydrate crystals, cholesterol crystals, silica crystals, aluminium salts and beta-amyloid aggregates[67, 72, 107-110].

Another mechanism described for the secretion of IL-1β from murine bone marrow derived macrophages is through exosomes, which are small vesicles of 30 to 100nm diameter with an endosomal origin[97, 101]. These exosomes contained IL-1β, caspase-1 and other inflammasome components and coalesced to form multivesicular bodies that fuse with the plasma membrane to allow release of IL-1β into the extracellular compartment[97].

Autophagy is a process that forms a double-layered membrane vesicle (autophagosome) that encloses specific cytosolic contents for degradation[111]. However, this mechanism has also recently been associated with IL-1β secretion. On one side, basal autophagy has an inhibitory role in the release of IL-1β by maintaining cellular homeostasis and avoiding the release of ROS and mitochondrial DNA, known activators of inflammasomes[112, 113]. Moreover, experiments on macrophages treated with TLR ligands and rapamycin have shown that pro-IL-1β is sequestered and destroyed inside of autophagosomes[114]. Conversely, other studies showed that starvation-induced autophagy in primary murine bone marrow-derived macrophages treated with nigericin increased the release of IL-1β through activation of NLRP3 inflammasome[115]. Other treatments that provoked IL-1β release via autophagy include alum, silica and amyloid-beta fibrils[115].

In recent years the formation of membrane pores has been established as a route of unconventional secretion of certain proteins, including IL-1β[47]. In macrophages the activation of caspase-4, -5, or -11 triggers a form of pro-inflammatory cell death called pyroptosis, through the cleavage of a cytosolic protein called gasdermin D[84, 85]. Gasdermin D is synthesized as a proform (molecular weight 53kDa), and after caspase processing the liberated N-terminal fragment (molecular weight 30kDa) binds to components of the plasma membrane such as phosphatidylinositol and phosphatidylserine, leading to formation of pores through which IL-1β is released[84, 85]. These studies also demonstrated that cells deficient in gasdermin D are resistant to pyroptosis after stimulation with canonical and non-canonical pathway ligands, and show a defect in IL-1 and IL-18 release[85].

Similarly, other studies have shown that the activation of the pseudokinase MLKL promotes potassium efflux, formation of the NLRP3 inflammasome and activation of IL-1β, while at the same time induces formation of pores in the cell membrane, like NT-gasdermin D, which enables release of IL-1β into the extracellular compartment[98]. This type of cell death mediated by MLKL is termed necroptosis[98].

Finally, Schroder and colleagues determined that the cleavage of pro-IL-1β in macrophages is sufficient to produce its direct secretion in slow manner. The authors hypothesised that the cleavage of pro-IL-1β by caspase-1 triggers a modification to the electric charges of the

molecule from negatively to positively charged[116]. This change promotes the binding of the resultant positively charged mature IL-1β to the projections and ruffles of the plasma membrane (areas of negative charge), leading to the translocation of this cytokine to the extracellular milieu[116].

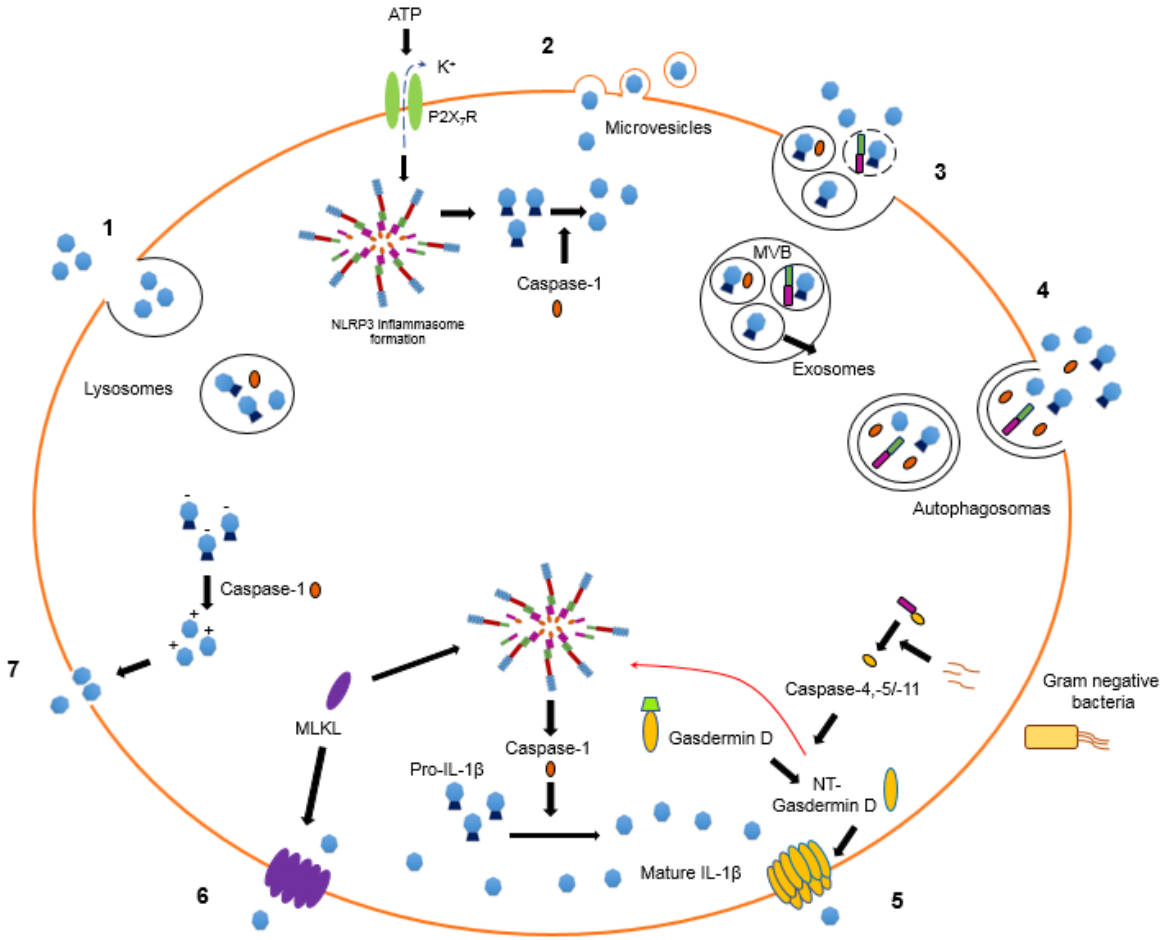


Fig. 7. Mechanisms of IL-1β secretion in immune cells. The multiple routes utilised by IL-1β to exit cells include exocytosis by lysosomes **(1)**, shedding of microvesicles **(2)**, secretion by exosomes **(3)** and autophagosomes **(4)**, release through pores formed by NT-gasdermin D **(5)** or MLKL **(6)**, and by translocation across the plasma membrane **(7)**. Adapted from *Rubartelli, 2018.*

1.3.6 IL-1β in VSMCs

IL-1β has mainly been studied in immune cells, with scant data about IL-1β synthesis, activation and secretion by VSMCs reported.

Initial investigations demonstrated that VSMCs express IL-1β in response to IL-1α, IL-1β or bacterial endotoxin[80, 117]. Other studies reported that pro-IL-1β is produced but not cleaved by VSMCs, due to the blocking effect of Serpin B9 on caspase-1 activity[118, 119]. However, this conclusion was based on coincubation experiments with recombinant proteins and subsequent western blot analysis. Later, Schönbeck and colleagues concluded that IL-1β is secreted by VSMCs in response to CD40 ligand stimulation, but these findings remain controversial[120]. More recent investigations report that VSMCs express, cleave and secrete IL-1β in response to a variety of stimuli, such as β-glycerophosphate, HMGB1, LLME, oxidized low-density lipoproteins, and calcium phosphate particles[121-125]. Nevertheless, in some of these studies the level of IL-1β in the conditioned media was negligible or could perhaps be attributed to inadvertent loss of plasma membrane integrity.

The results of these investigations will be explained and discussed in detail in the relevant introductions of the following chapters.

Hypothesis

I hypothesise that synthesis, cleavage and release of IL-1β from activated VSMCs drives vascular inflammation and disease.

2. Materials and methods

Materials

All materials were from Sigma Aldrich, unless stated otherwise

Reagent	Source	Identifier
Antibodies		
Rabbit polyclonal anti-human IL-1α	Peprotech	500-P21A
Goat polyclonal anti-human IL-1β	R&D	AF-201-NA
Rabbit Polyclonal Gasdermin D antibody	Proteintech	20770-1-AP
Bovine Polyclonal Anti-Goat HRP	Jackson Immunoresearch	805-035-186
Donkey Polyclonal Anti-rabbit HRP	GE Healthcare	NA934V
Recombinant Proteins, peptides and chemicals		
Human IL-1α	Peprotech	200-01A
Caspase-1	Enzo	BML-SE168
PDGF-β	Abcam	ab9706
TGF-β	Abcam	ab50036
IFN-γ	Peprotech	300-02
BMP-2	Peprotech	120-02
BMP-9	Peprotech	120-07
LPS	Invitrogen	tlrl-peklps
ATP	Enzo	ALX-480-021-G001
MSU	InvivoGen	tlrl-msu
Nigericin	Invivogen	tlrl-nig
Isohelenin	Insight	SC-221769
MCC950	InvivoGen	inh-mcc
QVD	Cayman	15260
ZVAD	Enzo	ALX-260-020-M001
YVAD	Enzo	ALX-260-154-R020
LEVD	Enzo	ALX-260-142-R020
IETD	Enzo	ALX-260-144-R100
VX-765	InvivoGen	inh-vx765i-1
BAY 678	Bio-Techne	5706/5
ZAAD	Abcam	ab142034
GM 6001	Sigma Aldrich	364205
MMP-2 Inhibitor I	Sigma Aldrich	444244
E-64	Sigma Aldrich	E3132

Dichloroisocoumarin	Enzo	BML-PI110-0010
Metformin	InvivoGen	tlrl-metf
Cycloheximide	Bio-Techne	0970/100
Anti-Fas Antibody clone CH11	Merck	05-201
Phorbol 12-myristate 13-acetate	Sigma Aldrich	16561-29-8
Brefeldin A	eBioscience,	00-4506-51
Protein G Dynabeads	Invitrogen,	10004D
NP-40	Sigma Aldrich	74385
Propidium iodide	Sigma Aldrich	P4170
Protein ladder	Biorad	161-0374
Coomassie	BioRad	161 -0787
ECL pierce 2 Western Blotting substrate	Thermo Scientific	80196
Streptavidin-PE	eBioscience	12-4317-87
Pierce™ TMB Substrate Kit	Thermofisher	34021
Sulfuric Acid	Fisher Chemical	J/8420/PB15
siRNA Non-Targeting	Dharmacon	D-001206-13-05
Smart pool siRNA Serpin B9	Dharmacon	M-015400-01-0005
Lipofectamine RNAimax	Invitrogen	13778030
Basic Nucleofector™ solution – Supplement reagent	Thermofisher	VPI-1004
RNAeasy mini kit	Qiagen	74104
ELISA Kits		
Human IL-1β plate-ELISA	R&D	DY201
Novex Life Technologies Luminex kits IL-1α	Invitrogen	LHC0811
Novex Life Technologies Luminex kits IL-6	Invitrogen	LHC0061
Others		
96-well MultiScreen 1.2µm filter plate	Merck Millipore	MSBVN1250
96-well EIA/RIA Plate	Corning	3590
PVDF membrane	Biorad	162-0177
X-ray film	Thermofisher	34089

Buffers and solutions

SDS PAGE and Western Blotting

Separating gel (15%)

5mL 30% acrylamide/Bis

2mL Tris 2M pH 8.8

3mL ddH_2O

50µL 20% SDS

50µL APS - ammonium persulphate - (100mg/mL)

25µL TEMED - N, N, N', N'-tetramethylethylenediamine -

Stacking gel:

3.9mL ddH_2O

0.7mL 30% acrylamide/Bis

0.6mL Tris 1M pH 6.8

25µL 20% SDS

25µL APS (100mg/mL)

8-10 µL TEMED

Cathode running buffer:

144g glycine

30g Tris

5g SDS

5L H_2O

pH 8.6

Anode running buffer:

30g Tris

5g SDS

5L H_2O

pH 8.6

Transfer buffer:

10 x buffer:

144g glycine

30g Tris

1L ddH_2O

pH 8.6

Blocking buffer:

5% milk in PBS

Bead diluent

1% BSA in PBS.

Plate ELISA

Blocking buffer

1% BSA in PBS.

Wash buffer

0.05% Tween®20 in PBS

Reagent diluent

0.1% BSA in PBS

Caspase-1 buffer

1mL HEPES (100 mM) pH 7.4

66µL 3M NaCl

200µL 0.2% CHAPS

4µL EDTA (0.5mM)

200mg glycerol

200µL DTT (100mM)

330µL H_2O

Methods

2.1 Cell culture

2.1.1 Origin of cell lines

Human aortic vascular smooth muscle cells (HASMCs) and human pulmonary smooth muscle cells (PSMCs) were isolated from aortas and pulmonary arteries of cardiac transplant patients, which were obtained by the Papworth Hospital Tissue Bank with full ethical consent. HeLa cells and Thp1 cells were obtained from ECACC.

Table 2. VSMCs utilised in the experiments

Cell type	Age of donor	Sex of donor	Passages utilised
HASMC	54	Female	6 – 12
HASMC	43	Female	6 – 13
HASMC	48	Male	7 – 12
HASMC	30	Male	7 – 13
PASMC	82	Male	6 – 9

To create the explants, vessels were washed with serum-free medium (Dulbecco's Modified Eagle Medium, high glucose -DMEM, Sigma, D5671- containing 10U/mL penicillin, 10mg/mL streptomycin and 5mg/mL L-glutamine), cut longitudinally, and the luminal side was gently scraped with a scalpel in order to remove the intima and expose the media where VSMCs are localised. The vessels were rinse intensively with serum-free medium and the media layer was carefully stripped off using bent forceps, placed in a new Petri dish and cut into ~1mm^2 pieces. The explanted pieces were transferred to 6-well plates and 1,5 mL of medium containing 20% foetal calf serum -FCS- (DMEM - Sigma, D5671-, FCS - Sigma, F7524- 10U/mL penicillin, 10mg/mL streptomycin and 5mg/mL L-glutamine) was added to the wells. The medium was changed every 3 – 4 days and single VSMCs spread out of the tissue samples after 2 – 3 weeks in culture. Once VSMCs reached confluency were subcultured.

2.1.2 Maintenance of cell lines

VSMCs and HeLa cells were cultured in DMEM (Sigma, D5671) containing 10% FCS (Sigma, F7524), 10U/mL penicillin, 10mg/mL streptomycin and 5mg/mL L-glutamine and passaged when at 80% confluence by detaching cells with trypsin. Thp1 cells were cultured in RPMI 1640 (Gibco, 31870-025) with 10% FCS, 10U/mL penicillin, 10mg/mL streptomycin, 5mg/ml L-glutamine and 2.3μM β-mercaptoethanol, and maintained at 3-9 x 10^5 cells/mL. Cells were incubated at 37°C with 5% CO_2.

2.1.3 Cell treatments

8 x 10^3 Aortic VMSCs or 1.5 x 10^4 Pulmonary VSMCs were plated per well in a 24-well tissue culture plate. Next day, cells were washed with PBS, media was replaced, and in some experiments cells were treated with human IL-1α neutralising antibody (0.5μg/mL). The following day, cells were washed again and incubated with IL-1α (20 ng/mL), LPS (1μg/mL), ATP (5mM), MSU (50μg/mL), Nigericin (15μM), isohelenin (10μM), MCC950 (20μM), QVD (20μM), ZVAD (20μM), YVAD (10μM), LEVD (10μM), IETD (2μM), VX-765 (10μM), BAY 678 (20μM), ZAAD (10μM), GM 6001 (10μM), MMP-2 Inhibitor I (20μM), E-64 (10μM), Dichloroisocoumarin (10μM, 20μM), PDGF-β (20 ng/mL), TGF-β (10 ng/mL), IFN-γ (10 ng/mL),

BMP-2 (100ng/mL), BMP-9 (100ng/mL), Cycloheximide (10µg/mL), Anti-Fas Antibody clone CH11 (100ng/mL), metformin (10mM), as indicated.

3×10^4 Thp1 cells were plated in a 24-well tissue culture plate and differentiated using 50ng/mL phorbol 12-myristate 13-acetate (PMA) for 2 days, then treated with LPS (1µg/mL), nigericin (15µM), MCC950 (20µM), QVD (20µM), ZVAD (20µM), YVAD (10µM), LEVD (10µM), IETD (2µM), VX-765 (10µM), as indicated.

After incubation, conditioned media was collected, clarified, and stored at -20 °C. Cells were washed with PBS, lysed using 0.5% NP-40 and collected with an equal volume of PBS. Cell lysates were subjected to 3 freeze-thaw cycles, centrifuged (4700g, 5mins) and supernatants stored at -20 °C. Samples were analysed by R&D Human IL-1β plate-ELISA.

2.1.4 HeLa bioassay

1.2×10^4 HeLa cells were plated in a 48-well tissue culture plate and incubated overnight. The VSMC or Thp1 lysate with the highest concentration of IL-1β was diluted with RPMI to obtain the same concentration of cytokine in both samples. Lysates only, or with IL-1α antibody (4µg/mL) and/or IL-1β antibody (4µg/mL) were pre-incubated for 15 minutes at room temperature, before adding to the cells for 6 hours. A negative control of media alone was included for each experiment. Conditioned media was collected, clarified and IL-6 concentration determined by bead ELISA.

In experiments using Brefeldin A, HeLa cells were plated as mentioned above. Next day, media was replaced and cells were treated with brefeldin A (dilution 1:1000) for 1 hour, before washing with PBS and re-addition media/brefeldin A (dilution 1:1000) to each well. VSMC and Thp1 necrotic lysates treated with IL-1β and/or IL-1α antibody as above, were added to cells and incubated for 5 hours. A negative control of media alone and media/brefeldin A was included for each experiment. Conditioned media was removed, cells were washed and then lysed with 50µL 0.5% NP-40 for 5 minutes before collection of the lysate with 50µL PBS to give a 1:2 dilution. The samples were clarified and supernatants analysed by bead ELISA to determine IL-6 concentration.

<u>VSMC necrotic lysate preparation</u>

8×10^5 VSMCs were plated in a T75 flask and incubated overnight at 37°C. Cells were washed with PBS, media replaced, and cells treated with IL-1α (20ng/mL) and/or LPS (1µg/mL) for 7 hours. Conditioned media was removed, cells harvested with trypsin and counted by flow cytometry (Accuri C6). VSMCs were resuspended in RPMI at a ratio of 1×10^6 VSMCs per

200µL RPMI and lysed through 3 freeze-thaw cycles using liquid nitrogen. The lysate was clarified and the supernatant stored at -20°C.

In experiments where lysate was immunodepleted, 60µL of magnetic Protein G Dynabeads were washed with PBS to remove storage buffer, and then resuspended in 60µL of PBS with 6µg of IL-1α antibody. The mix rolled at 40 rpm, RT for 45 minutes. ~200µL VSMC lysate was combined with 20µL of the magnetic beads/IL-1α antibody and rolled at 40 rpm, RT for 30 minutes, then the lysate was separated from the magnetic beads using a magnet, and placed into a new tube. The process was repeated twice. Finally, the IL-1β and IL-1α concentration in the VSMC lysate after immunodepletion was determined by ELISA.

Thp1 cell necrotic lysate preparation

2 x 10^5 Thp1 cells were plated in a 24-well tissue culture plate. 50ng/mL phorbol 12-myristate 13-acetate (PMA) was added to the media to cause differentiation of Thp1 monocytes into macrophages. After 2 days of incubation, Thp1 cells were washed with PBS, the media was replaced and cells were treated with LPS (1µg/mL) for 3 hours and then nigericin (15µM) and LPS for 3 hours. Cells were harvested and resuspended into the conditioned media, and subsequently lysed through 3 freeze-thaw cycles using liquid nitrogen. Lysates were clarified and the supernatant stored at -20°C. IL-1β concentration was determined by ELISA.

2.1.5 Cell transfection

Serpin B9 knock down

8 x 10^3 VMSCs were plated in a 24-well tissue culture plate. Next day, cells were washed with PBS and treated with IL-1α neutralising antibody (0.5µg/mL). The following day cells were washed with PBS, media was replaced, and cells were transfected with non-targeting siRNA or smart pool siRNA Serpin B9 (10pmol) using lipofectamine RNAimax following manufacturers' protocols. 48 hours later cells were washed with PBS, and treated with IL-1α (20ng/mL) for 7 hours. Then, conditioned media was collected and clarified, and supernatants were stored at -20 °C. Cells were washed with PBS, lysed using 0.5% NP-40 and collected with PBS to give a 1:2 dilution. Cell lysates were subjected to 3 freeze-thaw cycles, centrifuged and supernatants stored at -20 °C. Samples were analysed by ELISA to determine concentration of IL-1β.

NT-Gasdermin D transfection

VSMCs were washed and resuspended into Basic Nucleofector™ solution and supplement reagent at a ratio of 4.5:1, respectively. 200µL of cell suspension corresponding to 1.2 x 10^6 VSMCs was combined with 4µg pcDNA3 or 4µg NT-Gasdermin-pcDNA3. 100µL of cell/DNA suspension was transferred into a cuvette and electroporated using program A-033

(NucleofectorTM 2b Device). 1mL of warm media was used to collect the sample and cells were seeded into 1 T25 flask containing 5mL of 10% DMEM. 24 hours after transfection cells were washed with PBS, media was replaced, and cells were treated with IL-1α (20ng/mL) for 6 hours, as indicated. After treatment conditioned media was collected, clarified and the supernatants were analysed by ELISA to determine concentration of IL-1β.

Transfection of IL-1β

VSMCs were washed and resuspended into Basic Nucleofector™ solution and supplement reagent at a ratio of 4.5:1, respectively. 100µL of cell suspension was combined with pcDNA3, p31- or p17-IL-1β-pcDNA3, as indicated. Then, the cell/DNA suspension were transferred into a cuvette and electroporated using program A-033. Warm media was used to collect the sample and cells seeded in duplicate in a 24-well tissue culture plate (6 x 10^4 cells/well). 24 hours or 30 hours after transfection, conditioned media was collected, clarified and the supernatants were analysed by ELISA to determine concentration of IL-1β.

LPS transfection

VSMCs were plated in 6 T75 flasks. 2 days later, cells were washed with PBS, media was replaced and 3 flasks were treated with IL-1α (20ng/mL) and LPS (1µg/mL). 15 hours later cells were trypsinised and pooled. Then, control and treated VSMCs were resuspended into Basic Nucleofector™ solution and supplement reagent at a ratio of 4.5:1, respectively. 100µL of cell suspension was combined with vehicle or 2µg LPS. Suspensions were transferred into a cuvette and electroporated using program A-033. Warm media was used to collect the sample and cells were seeded in quadruplicate in a 24-well tissue culture plate (6 x 10^4 cells/well). Conditioned media was collected immediately after transfection and 6 hours later, clarified and supernatants analysed by ELISA to determine concentration of IL-1β.

2.2 Measurement of cell death

Floating and attached cells were pooled and incubated with propidium iodide (1µg/ml) for 5 minutes. The fluorescence was analysed by flow cytometry using the FL-2 channel.

2.3 Caspase-1 cleavage assay

Conditioned media from activated VSMCs or ~60ng of pro-IL-1β (purified in our lab) was added to caspase-1 buffer and treated with 100U caspase-1 or H_2O (control). Samples were incubated for 1 hour at 37°C, diluted 1:100 and analysed by plate ELISA to measure concentration of cleaved IL-1β.

2.4 Protein detection

2.4.1 Western blotting

Pelleted cells were resuspended into 3x Laemmli buffer at a volume that gave a cellular density of 1 x 10^5 VSMCs or 5 x 10^5 Thp1 cells in each 15µL of sample. 15µL of lysates was loaded onto each lane of an SDS PAGE gel (15% separation), and run alongside 10µL of protein ladder. The SDS gel was run at 15mA until the dye reached the bottom of the gel. The SDS gel was equilibrated using transfer buffer and blotted onto PVDF membrane at 85mA per gel for 2 hours. The remnant gel was stained with Coomasie and dried. The membrane was blocked in 5% milk (Marvel) for 1 hour and then incubated overnight at 4°C in 5% milk (Marvel) with anti-IL-1β (1:500) or anti-Gasdermin D (1:200 or 1:600). Next day, the membrane was washed 3 times in PBS/Tween 0.05% for 5 minutes, and incubated with a secondary antibody – Bovine Anti-Goat HRP (dilution 1:2000) or Donkey Anti-rabbit HRP (dilution 1:2000), respectively - for 1 hour at room temperature. The membrane was washed 3 times in PBS/Tween 0.05%, then incubated in ECL pierce 2 Western Blotting substrate and exposed to X-ray film.

2.4.2 Bead ELISA

IL-1α and IL-6 were measured using Novex Life Technologies Luminex kits. Per well of a 96-well MultiScreen 1.2µm filter plate, 1.25µL antibody beads, 5µL biotinylated secondary antibody and 20µL FACS buffer was added to 25µL of sample or serial dilutions of standards (IL-1α 21,000 to 0pg/mL, IL-6 20,000 to 0pg/mL) and incubated for 2 hours at room temperature in the dark with agitation. Wells were washed twice with 200µL FACS buffer, and then 50µL streptavidin-PE (0.2µg/mL) was added per well and incubated for 1 hour at room temperature in the dark with agitation. Wells were again washed twice with 200µL FACS buffer. Then, beads contained in each well were resuspended in 200µL FACS buffer and analysed by flow cytometry (Accuri C6). The average PE fluorescence of 300 events were measured and recorded and cytokine concentrations were calculated through a standard curve using a 4 parameter logistic fit.

2.4.3 Plate ELISA

Concentrations of IL-1β was determined using R&D DuoSet® ELISA Development system kit. Wells of a 96-well EIA/RIA Plate were coated with 400ng capture antibody diluted in PBS overnight. The plate was aspirated and washed four times with wash buffer, and then incubated with blocking buffer for 1 hour. The washing step was repeated and the samples and serial dilutions of standards (2,000 to 0pg/ml) were added to the plate. After 2 hours, the plate was aspirated and washed, and then 20ng of detection antibody diluted in reagent diluent was added to each well and incubated for 2 hours. The plate was washed again and each well incubated with 100µL Streptavidin-HRP diluted (1:40) in reagent diluent for 20 minutes at room

temperature in the dark with agitation. The washing step was repeated once more, and each well was incubated with 100µL Substrate solution/well (Pierce™ TMB Substrate Kit) for ~3 minutes until an even colour separation was obtained across the standards. Finally, 100µL Stop solution/well (Sulfuric acid) was added and the optical density determined using a microplate reader (BMG Fluorstar; wavelength 450 nm).

2.5 Protein purification

Human p31 IL-1β was cloned into pGEX-4T-3 (GE). For GST purification, IPTG-induced cultures (Rosetta) were lysed in 50 mM Na2HPO4 pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, benzonase, lysozyme, and protease inhibitors and clarified by centrifugation (5,525 g, 1 hr, 4°C). Supernatants were loaded onto a GSTrap column (GE), washed with PBS; DTT 1 mM; EDTA 1 mM and eluted in Tris 50 mM; NaCl 100 mM; DTT 1 mM; reduced L-glutathione 50 mM; adjusted to pH 8 using an FPLC system (AKTA pure). Protein was dialysed overnight using Tris 10 mM, pH8; NaCl 50 mM and stored in 10% glycerol (-80°C). Determination of protein concentration and purity was carried out by Coomassie staining (G-250; Bio-Rad) and quantification with an Odyssey scanner (Licor).

2.6 Gel filtration

Size fractionation was performed on an AKTA PURE FPLC system (GE Healthcare Life Science). Activated VSMCs harvested with trypsin were resuspended in PBS and lysed through 3 freeze-thaw cycles, samples cleared with centrifuged (9600g, 5mins) and 100µL of supernatant injected onto the column (Superdex 75). PBS was used as elution buffer and fractions of 1 mL were collected at a flow rate of 0.7mL/min. Total eluent was collected in 73 fractions and the concentration of IL-1β in each was determined by ELISA.

2.7 Molecular biology

2.7.1 RNA extraction and purification

Purification of RNA from VSMCs was performed with an RNAeasy mini kit (Qiagen 74104) following manufacturers' protocols. Briefly, VSMCs were washed with PBS, lysed directly in wells using 350µL of RLT buffer and transferred to Eppendorf tubes. 1 volume of 70% ethanol was added to each sample. RLT buffer-ethanol mix was transferred to an RNeasy spin column placed in a 2ml collection tube and centrifugated for 15 seconds at 8,000g. 700µL of buffer RW1 was added to wash the membrane and the RNeasy spin column was centrifugated for 15 seconds at 8,000g. Then, 500µL of buffer RPE was added to the RNeasy spin column and centrifuged for 15 seconds at 8,000g and the last step was repeated but centrifuged for 2 minutes at 8,000g. Finally, the RNeasy spin column was placed in a new 1.5mL collection tube and 30µL of RNase-free water was added directly to the membrane and centrifuged for 1 minute at 8,000g to elute the RNA.

2.7.2 RNA reverse transcription

For each sample the following mix was prepared in a microcentrifuge tube, incubated for 10 minutes at 70°C, centrifuged at 500g for 5 seconds and put on ice:

9.4µL RNA (~1µg)
1µL OligoDT (0.5µg/mL)
0.5µL Random primers (0.5µg/mL)
2µL dNTPs (10mM)

A second mix was made and added to each tube:

4µL $MgCl_2$ (25mM)
2µL 10x buffer
0.5µL Recombinant RNAsin ribonuclease inhibitor (40U/µL)
0.6µL AMV reverse transcriptase 0.65µl (25U/µL)

The samples was incubated at 25°C for 10 minutes, 42°C for 50 minutes, 95°C for 5 minutes and 4°C for 5 minutes before storage at -20°C.

2.7.3 qPCR

1µL cDNA was mixed with the following Promega reagents and TaqMan probes:
2µL 10X Amplitaq Gold buffer
1.6µL 25mM $MgCl_2$
0.4µL 10mM dNTPs
0.1µL Amplitaq enzyme
13.9µL H_2O
1µL Taqman Probe:

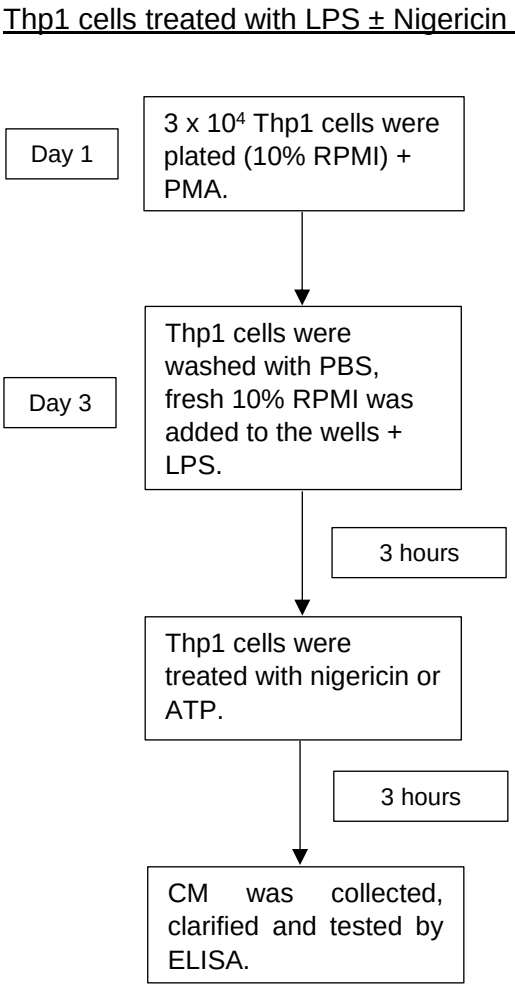
Gene	**Catalogue number (Life technologies)**
GusB	Hs00939627_m1
TBP	Hs00427620_m1
Caspase 1	Hs003544836_m1
Caspase 4	Hs01031951_m1
Caspase 5	Hs00362078_m1
Caspase 8	Hs0101815_m1
Gasdermin D	Hs00986739_g1

The mix was incubated in a thermocycler (Qiagen RotorGene 6000) using a two-step program of 95°C for 10mins followed by 95°C for 15 secs and 60°C for 30 secs, for 40 cycles. The data was evaluated using RotorGene 6000 series software 1.7 and fold changes calculated by the Delta Delta CT method.

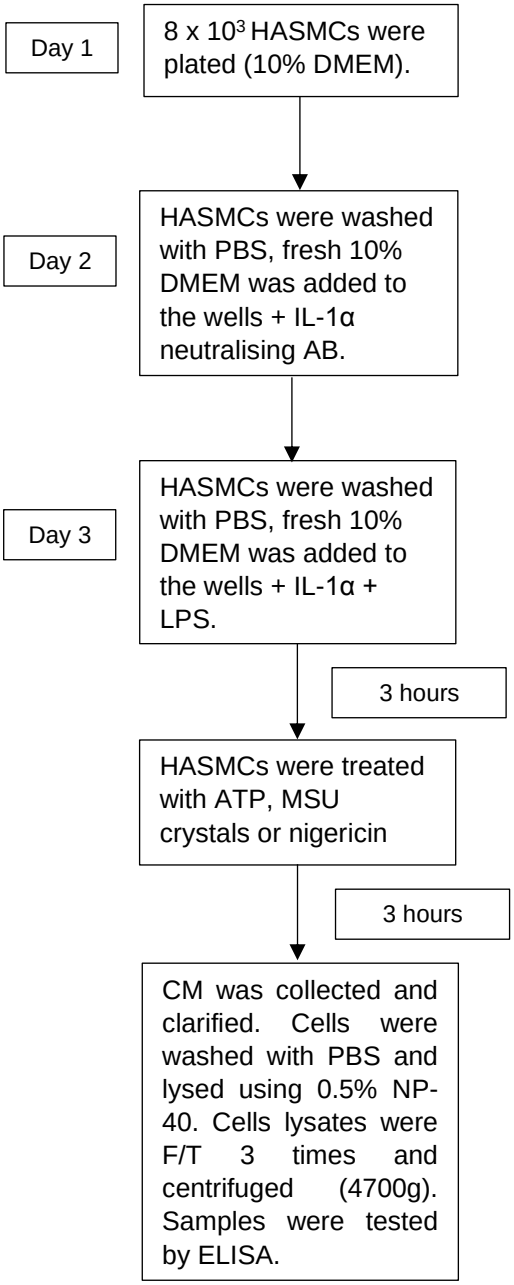
2.8 Statistics.

Statistical analyses were performed using GraphPrism. Data are expressed as mean ± standard error of the mean (SEM), unless otherwise stated. Unpaired t-test was used to compare the means of two conditions. One-way ANOVA was utilised in experiments with 3 or more conditions, with Dunnett's post-hoc used to compare every mean with the control and Tukey's post-hoc test to compare every mean to every other mean. Threshold for significance was set at $p \leq 0.05$. n = individual biological replicates performed in duplicate on different days.

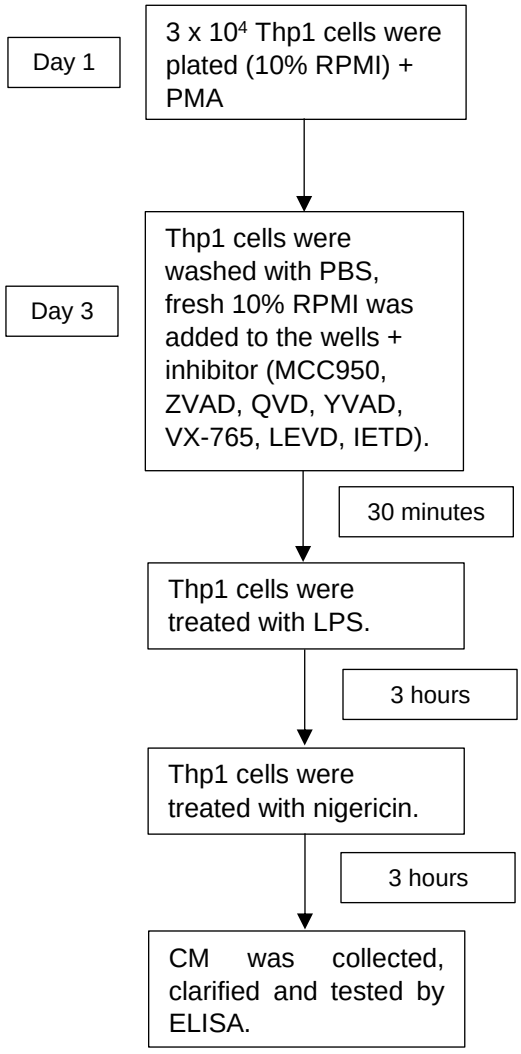
2.9 Schematic diagrams of the experimental procedures



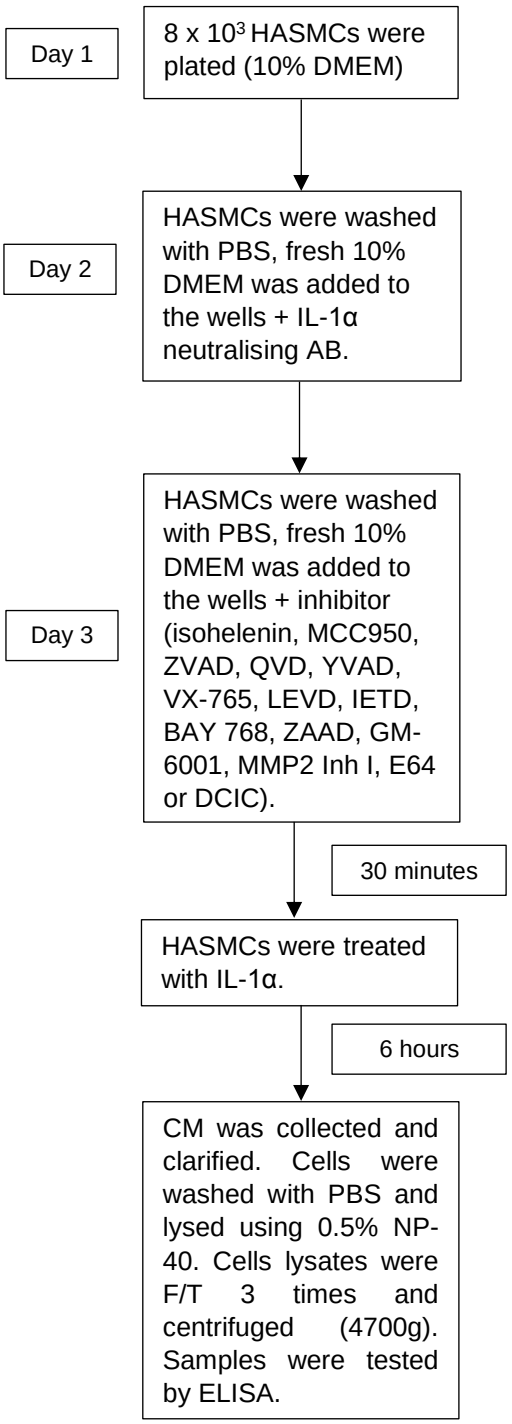
HASMCs treated with IL-1α+LPS ± common NLRP3 inflammasome activators



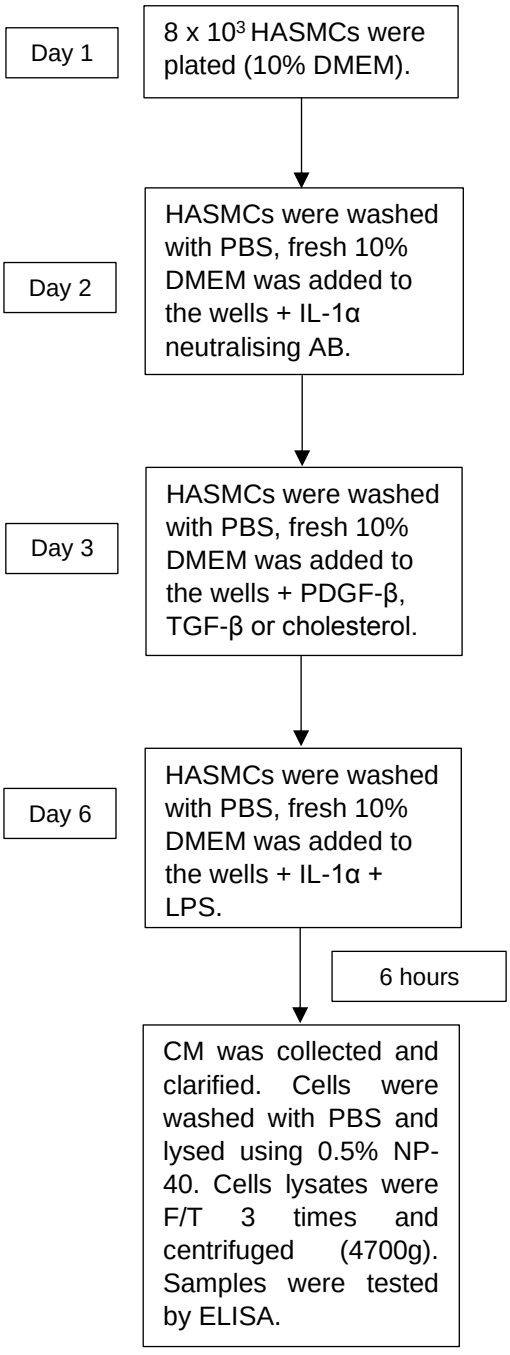
Thp1 cells treated with LPS + Nigericin ± inhibitors



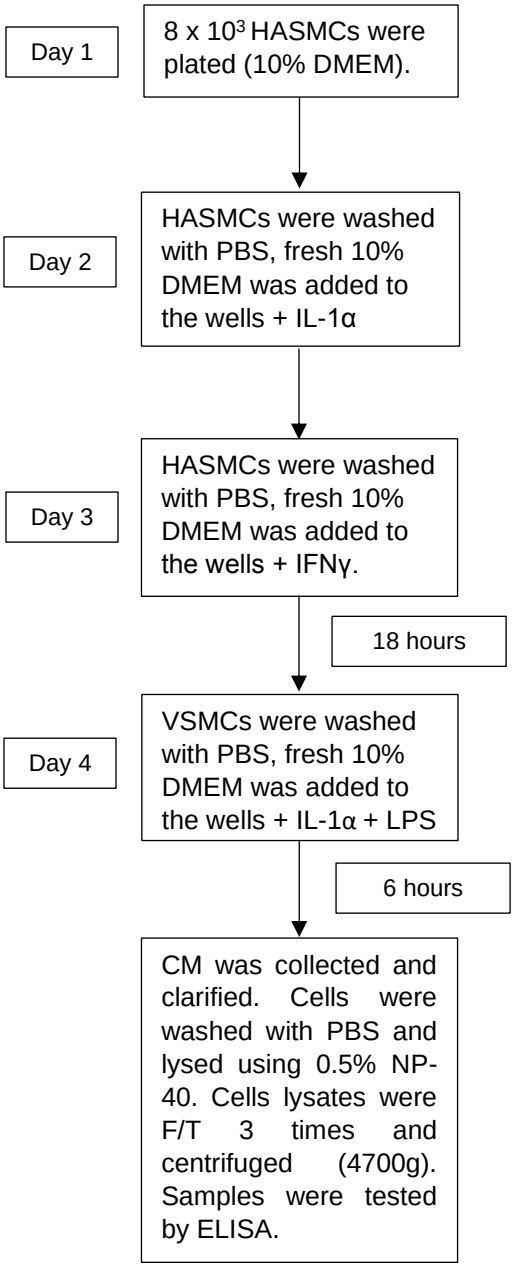
HASMCs treated IL-1α ± inhibitors



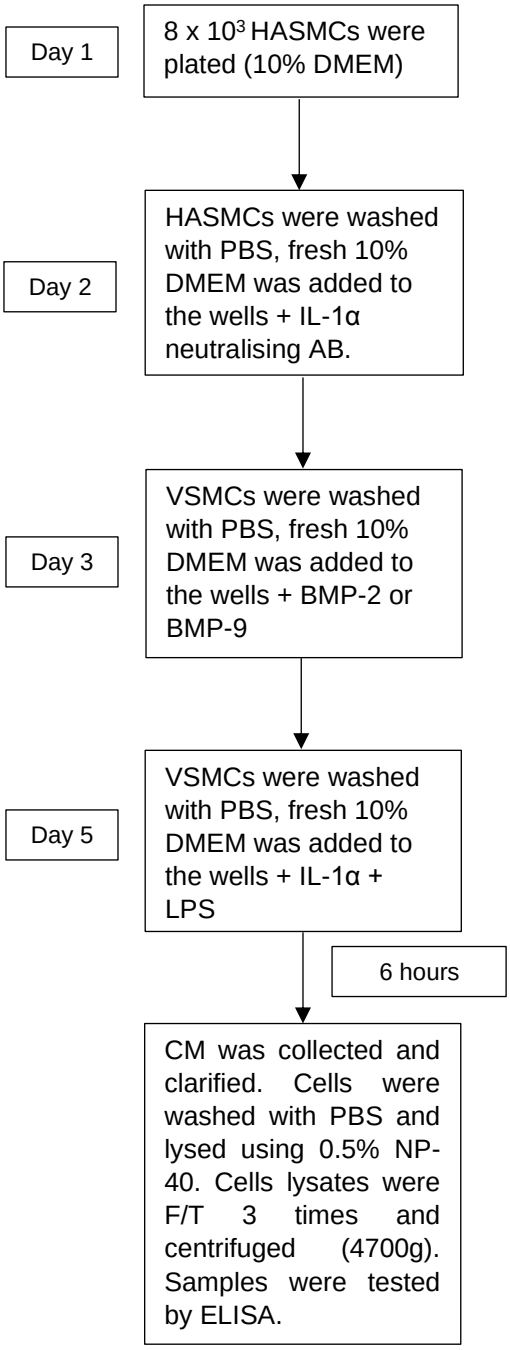
HASMCs treated with IL-1α+LPS ± inducers of phenotypic change



HASMCs treated with IL-1α+LPS ± inducers of phenotypic change



HASMCs treated with IL-1α+LPS ± inducers of phenotypic change



3. IL-1β is processed by Vascular Smooth Muscle Cells

Introduction

VSMCs are crucial for both the progression and stabilization of atherosclerotic plaques[126-129]. Likewise, numerous studies have shown that IL-1β, a central mediator of inflammation, plays a key role in atherogenesis (see chapter 1). In the late 80's, pioneering investigations by Libby and collaborators demonstrated that VSMCs stimulated with IL-1α, IL-1β and/or bacterial endotoxin express the IL-1β gene and secrete biologically active IL-1, which was determined by the capacity of the conditioned media to induce thymocyte proliferation[80, 117]. However, they discovered later, using a more specific IL-1 bioassay, that VSMCs do not release IL-1 and that the previous thymocyte coactivation activity was due to IL-6[130]. Interestingly, protein expression of IL-1β in VSMCs was not analysed in any of these first studies.

Subsequently, new investigations were performed to study the synthesis and activation of IL-1β in VSMCs. Schönbeck and collaborators found that coincubation of recombinant pro-IL-1β with conditioned media or cell lysates from non-stimulated or TNF-α-stimulated VMSCs did not lead to the appearance of mature IL-1β in Western blot analysis[118]. Therefore, they examined the gene and protein expression of caspase-1 to establish if IL-1β was not processed due to the lack of this enzyme, but the results showed that VSMCs possess caspase-1[118]. Finally, they explained the finding, based on Western blot results, through the existence of a caspase-1 inhibitor in VSMCs that in a concentration-dependent manner prevents the cleavage and subsequent release of recombinant IL-1β[118].

Later, the group of Schönbeck identified serpin proteinase inhibitor 9 (serpin B9) as the protein that constitutively blocks IL-1β processing in VSMCS[119]. This conclusion was formulated based on the inhibition of recombinant pro-IL-1β cleavage when caspase-1 was preincubated or incubated in parallel with recombinant serpin B9, and that the inhibition was abolished when VSMC lysates were previously immunodepleted of serpin B9[119]. Furthermore, unstimulated and TNF-α stimulated VSMCs treated with serpin B9 antisense oligonucleotides increased concentrations of IL-1β in conditioned media, as determined by ELISA, from basal levels of approximately 800 pg/mL to 2500 pg/ml after reduction of serpin B9 expression[119]. Likewise, immunohistochemistry studies showed a profuse, uniform and constitutive expression of serpin B9 and absence of IL-1β in normal arteries, in contrast to low expression of serpin B9 and high expression of IL-1β in atherosclerotic arteries[119].

Concerning these two studies, it is important to highlight that conclusions were drawn based on results of coincubation of recombinant proteins analysed by Western blot, which requires a higher concentrations of antigen to produce a visible signal compared to other methods such as ELISA[131]. Only conditioned media of VSMCs exposed to antisense oligonucleotide serpin

B9 was tested by ELISA and in contradiction to the final analysis of the data, high concentrations of mature IL-1β were detected in all groups including the control.

Another study by Schönbeck and collaborators reported a different response when VSMCs were treated with recombinant CD40 ligand[120]. In this case, VSMCs stimulated with CD40 ligand increased expression of pro-IL-1β and activated caspase-1[120]. Also, they concluded that CD40 ligand stimulation lead to generation and secretion of active mature IL-1β, based on the increased cell proliferation in the thymocyte assay. However, the presence of mature IL-1β in the conditioned media or cell lysates of VSMCs could not be corroborated by either radioimmunoprecipitation assay or Western blot analysis, as the authors reported[120].

Therefore, in the light of this conflicting data set, we considered it pertinent to reinvestigate basic aspects of synthesis, cleavage and release of IL-1β from VSMCs using diverse techniques, including ELISA-based analysis. This chapter shows our initial observations and the multiple experiments carried out to confirm or exclude other potential factors or situations that could artefactually cause these initial results. Intriguingly, we find that IL-1β is readily expressed and activated after treatment of VSMCs with diverse stimuli. However, IL-1β accumulates inside the VSMC and is not released.

Aims:

1. To study the effect of different stimuli on the production and processing of IL-1β by VSMCs.

2. To evaluate if IL-1β is release to the conditioned media by activated VSMCs.

3. To determine if VSMCs isolated from different anatomical regions respond to stimulation in a different manner.

3.1 IL-1β is synthesised and processed by HASMCs.

To investigate IL-1β production and activation in HASMCs, cells were treated with IL-1α and/or LPS and protein expression in whole cell lysates analysed by western blotting and ELISA. These initial results confirmed that resting HASMCs contained pro-IL-1β and that IL-1α and/or LPS treatment led to increased pro-IL-1β expression and accumulation of the mature form of IL-1β (p17) in VSMCs within 7 hours (Fig. 8A,B). The expression and cleavage of pro-IL-1β was superior in HASMCs treated with IL-1α compared to LPS, and a synergistic effect was observed in HASMCs treated with both reagents (Fig. 8A,B).

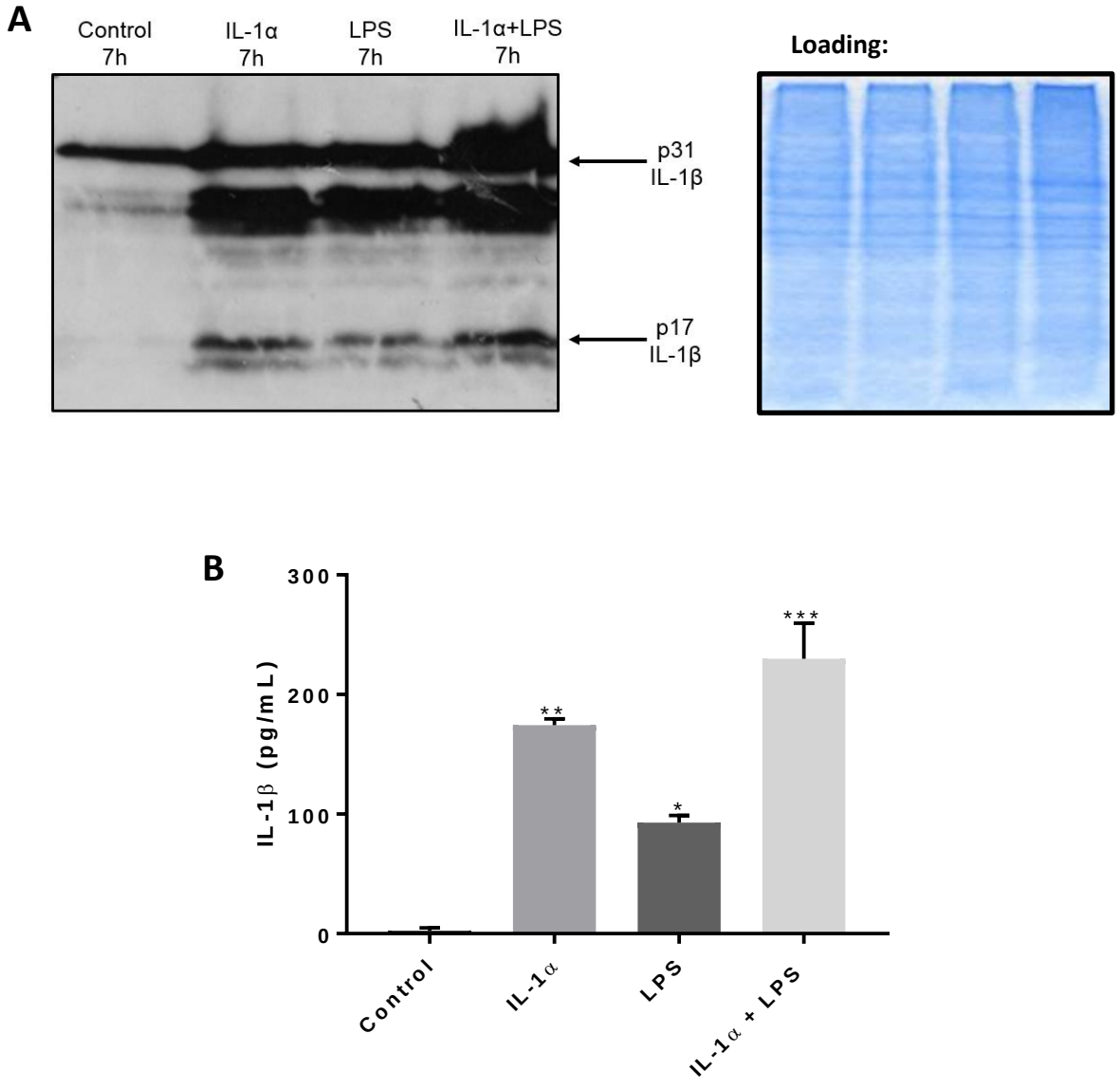


Fig. 8. HASMCs produce and cleave of IL-1β after IL-1R1 and/or TLR4 ligation. (A) Western blot for IL-1β in HASMC lysates after treatment with IL-1α and/or LPS (7 h), as indicated. **(B)** ELISA data showing the level of IL-1β inside HASMCs after treatment with IL-1α and/or LPS (7 h), as indicated. Data represents mean ± SEM, n = 2 (A), n = 6 (4 independent donors) (B) ; p = ns > 0.05, * ≤0.05, **≤ 0.01, ***≤0.001.

3.2 IL-1β is not detected in the conditioned media of HASMCs after ligation of IL1R1 and/or TLR4 receptor.

Cleavage of IL-1β normally leads to its release from the cell[132]. Therefore, I investigated if IL-1β was secreted after stimulation of HASMCs by testing the conditioned media (CM) by ELISA. This showed that HASMCs activated with IL-1α and/or LPS did not release IL-1β into the CM (Fig. 9).

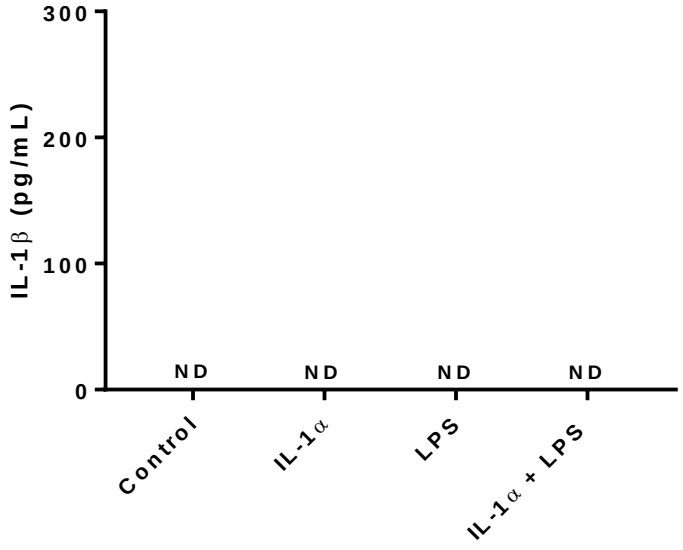


Fig. 9. IL-1β is not found in the CM of HASMCs after IL-1R1 and/or TLR4 ligation. ELISA data displaying the level of IL-1β in the CM of HASMCs treated ±IL-1α and/or ±LPS (7 h), as indicated. n = 5; ND = not detected.

3.3 Mature p17 IL-1β is the main form recognised by R&D IL-1β ELISA Kit.

One possibility for the lack of IL-1β in the CM of activated HASMCs is that the ELISA used did not detect the mature form. To investigate this, I directly tested recombinant forms of p17 and p31 IL-1β that were purified in our laboratory. The results displayed that the ELISA kit mainly measures the cleaved p17 form of IL-1β (Fig. 10 A), and that it has a very low sensitivity (~10 fold lower) to detect the full-length p31 form of IL-1β (Fig. 10 B). Simultaneously, SDS-PAGE and coomassie staining was performed and I confirmed the integrity of the p31 IL-1β that was analysed by ELISA (Fig. 11).

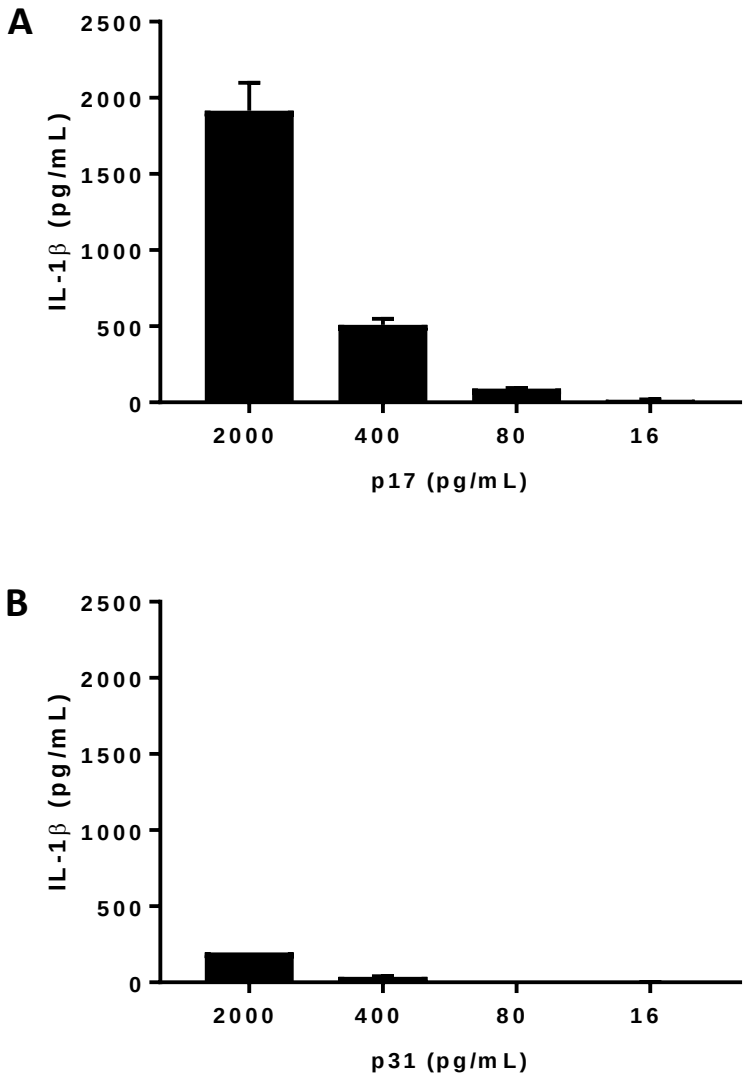


Fig. 10. R&D ELISA kit is optimal for detecting the cleaved form of IL-1β. (A,B) ELISA data showing detection of different concentrations of p17 IL-1β **(A)** and p31 pro-IL-1β **(B).** Data represents mean ± SEM, n = 3 (A,B).

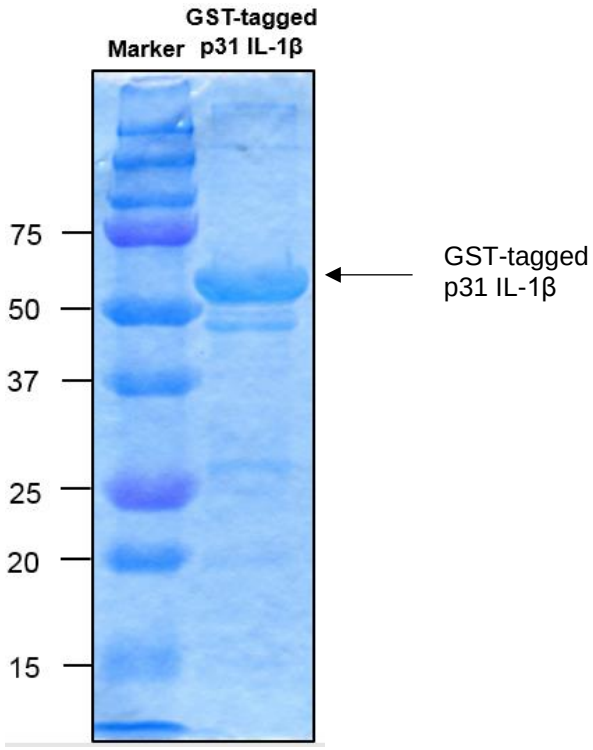


Fig. 11. p31 IL-1β purified in the laboratory maintains structural integrity. SDS-PAGE and coomassie staining of p31 IL-1β purified in the lab.

3.4 Pro-IL-1β is not released into the conditioned media by HASMCs.

After confirming that the R&D ELISA kit measures mainly the mature form of IL-1β, I wondered whether HASMCs secrete only pro-IL-1β and hence it is not detected in the CM. To answer this question, CM from activated HASMCs was treated with caspase-1, which should cleave pro-IL-1β to the mature form that can be detected by the ELISA. I also used pro-IL-1β purified in our laboratory as a positive control for caspase-1 activity. This showed caspase-1-dependent production of mature IL-1β from recombinant pro-IL-1β (Fig. 12), but no generation of mature IL-1β from activated HASMC CM (Fig. 12). This suggests that pro-IL-1β is not released by activated HASMCs. However, other experiments could be carried out to evaluate the presence of pro-IL-1β in the CM, for example, proteins and extracellular vesicles in the CM of activated VSMCs could be concentrated by ultrafiltration (using centrifugal filters) or ultracentrifugation, followed by determination of IL-1β by Western blotting.

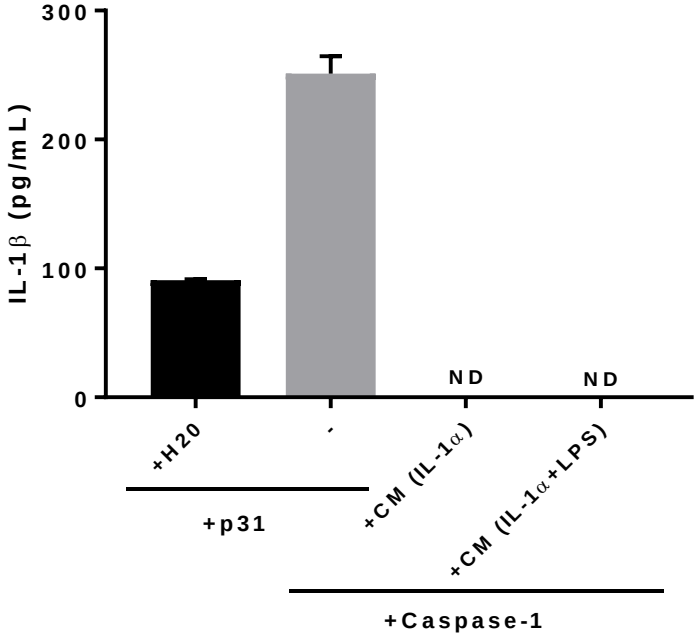


Fig. 12. Activated HASMCs do not release pro-IL-1β. ELISA data indicating the level of mature IL-1β after incubation of pro-IL-1β or CM from IL-1α or IL-1α/LPS (7 h) stimulated HASMCs with caspase-1. n= 2; ND = not detected.

3.5 IL-1β is not secreted through microvesicles, exosomes or lysosomes.

Microvesicles, exosomes and lysosomes have all been described as a mechanism for secretion of IL-1β in other cell types[103-105]. Thus, I studied whether IL-1β is secreted from activated HASMCs through the formation of any of these vesicles, and hence whether IL-1β is hidden from detection by the ELISA. To test this, CM from HASMCs treated with IL-1α and/or LPS for 7 hours (known to be enough time for HASMCs to synthesise and cleave IL-1β - Fig 8) was treated with Nonidet P-40 (NP-40) detergent in order to lyse the membrane of the microvesicles, exosomes or lysosomes and enable contact of IL-1β with the ELISA antibodies. This experiment still showed an absence of IL-1β in activated HASMC CM, suggesting IL-1β is not inside a microvesicle, exosome or lysosome (Fig. 13).

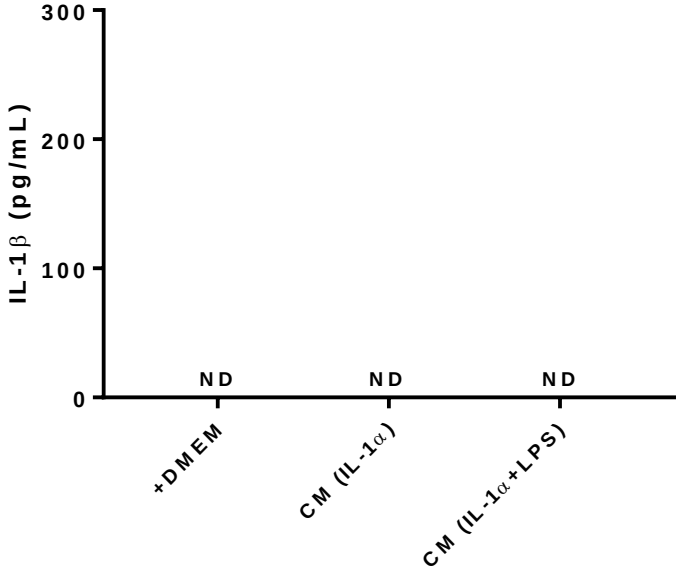


Fig. 13. Secretion of IL-1β from HASMCs is not via formation of microvesicles, exosomes or lysosomes. ELISA data showing concentrations of IL-1β in CM from HASMCs treated with IL-1α and/or LPS (7 h) as indicated, and CM treated with NP-40 detergent. n= 3; ND = not detected.

3.6 Mature IL-1β is detected in the conditioned media of monocytic cells.

Thp1 cells are an immortal monocytic cell line[133]. Nigericin and LPS treatment induces inflammasome activation and IL-1β production, cleavage and release from these cells[134, 135]. Therefore, I used Thp1 cells as a positive control for IL-1β release. This confirmed that Thp1 cells synthesise and process IL-1β, as well as secrete the cytokine into the CM after treatment, as expected (Fig. 14A,B). Furthermore, this experiment demonstrates that the R&D ELISA kit can properly measure cell-derived mature IL-1β in the CM, ruling out any detection issue related to the kit.

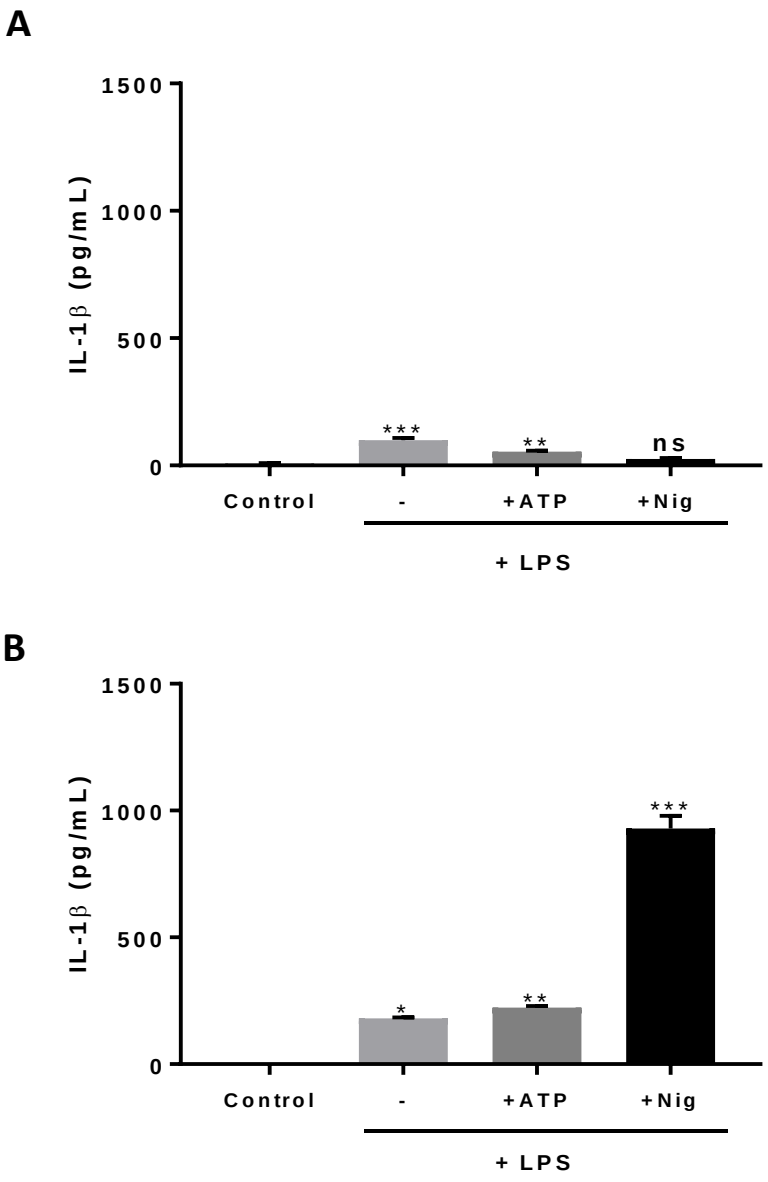


Fig. 14. IL-1β is cleaved and secreted from Thp1 cells after inflammasome activation. (A,B) ELISA data showing the level of mature IL-1β inside **(A)** and in the CM **(B)** of Thp1 cells treated ± ATP (3 h), ± nigericin (3 h), or ± LPS (6 h) as indicated. Data represents mean ± SEM, n = 3 (A,B); p = ns > 0.05, * ≤0.05, **≤ 0.01, ***≤0.001.

3.7 Production and cleavage of IL-1β is time-dependent.

I also investigated the effect of IL-1α treatment on HASMCs over time. Western blotting showed a time-dependent increase in the expression of pro-IL-1β (p31) and progressive accumulation of mature IL-1β (p17) in HASMCs over time (Fig. 15). Interestingly, after 24 hours of incubation with IL-1α the expression of pro-IL-1β decreased and mature IL-1β was not detectable (Fig. 15). The reason for these findings could be the inactivation of IL-1α signalling after prolonged culture, with degradation of pro-IL-1β, or perhaps release of mature IL-1β from HASMCs between 8 - 24 hours of stimulation. Therefore, further studies are required to investigate the apparent disappearance of intracellular IL-1β at 24 hours.

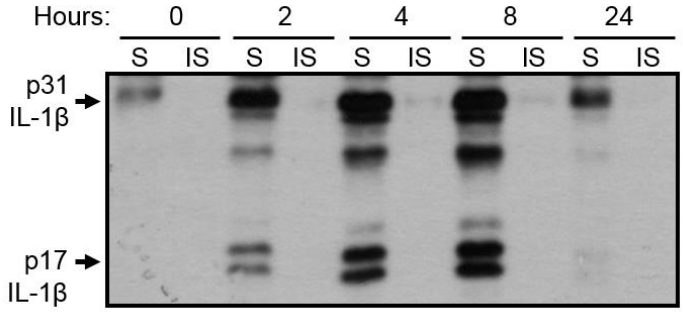


Fig. 15. IL-1β is synthesised and processed in a time-dependent manner in HASMCs. Western blot for IL-1β in HASMC lysates after treatment with IL-1α for the time indicated. Representative of n = 2; S = soluble, IS = insoluble fraction. This experiment was performed in collaboration with Sarah Gardner.

3.8 IL-1β is absent in the conditioned media of HASMCs treated with common inflammasome activators.

Our data indicates that although HASMCs cleave IL-1β in response to IL-1α and/or LPS treatment, they do not secrete it. However, to study if this phenomenon was stimulus-dependent, HASMCs were incubated with ATP (P2X7 ligand), MSU crystals or nigericin (potassium ionophore) - all well characterised NLRP3 inflammasome activators that cause IL-1β release from immune cells[132]. I found that activated HASMCs did not secrete IL-1β in response to any of these treatments (Fig. 16A,B,C). Additionally, I observed that ATP and MSU crystals had no significant effect in the production of mature IL-1β by HASMCs (Fig. 16D,E). In contrast, nigericin decreased the ability of the cells to generate IL-1β (Fig. 16F) which was not explained by a reduction in the number of viable cells (Fig. 17), and could be caused by inhibition of protein synthesis, an effect of some potassium ionophores reported previously in other cell types[136, 137]. These results suggest that only a single stimulus is required to trigger the cleavage of IL-1β by HASMCs and the resultant mature IL-1β is not release into the conditioned media despite the exposure of cells to a second stimulus.

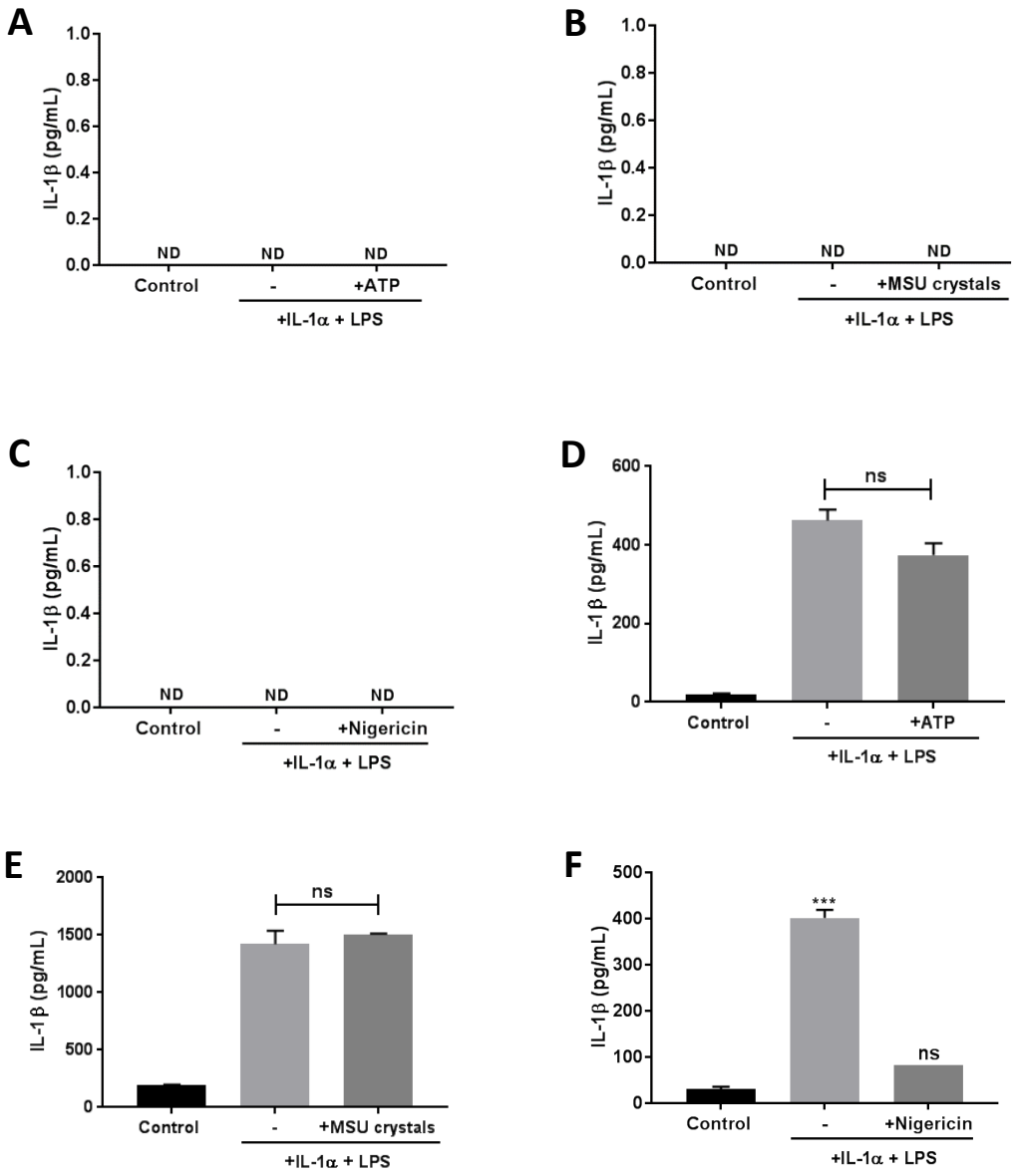


Fig. 16. Known inflammasome activators do not lead to IL-1β release from HASMCs. (A-F) ELISA data indicating the level of mature IL-1β in the CM **(A-C)** or inside of HASMCs **(D-F)** treated ± IL-1α and LPS (6 h), ± ATP (3 h), MSU crystals (3 h), or nigericin (3 h), as indicated. ND = not detected. Data represents mean ± SEM, n = 3 (A, C, D, F), n = 5 (B, E); p = ns > 0.05, * ≤0.05, **≤ 0.01, ***≤0.001.

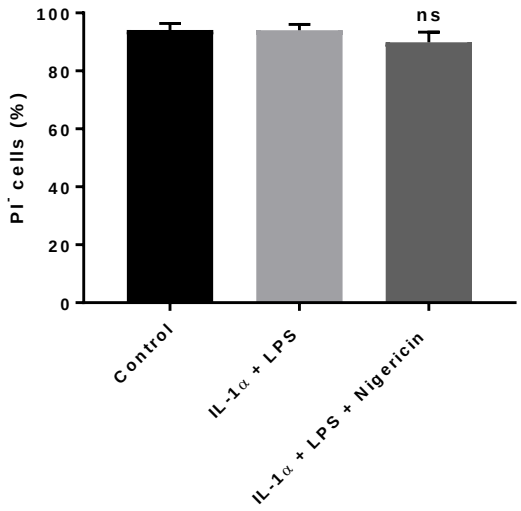


Fig. 17. Treatment with IL-1α, LPS and/or nigericin does not cause death of HASMCs. Flow cytometry data showing the percentage of viable cells (PI -ve) after treatment ± IL-1α and LPS (6 h), ± nigericin (3 h), as indicated.

3.9 Pulmonary smooth muscle cells do not secrete IL-1β.

I investigated IL-1β in SMCs from pulmonary artery (PSMCs) in order to know if SMCs isolated from a different location and exposed to less mechanical stress, as a consequence of lower blood pressure[138], can synthesise, process and secrete IL-1β. I observed that PSMCs treated with IL-1α synthesised and cleaved IL-1β, but the concentrations were lower compared to HASMCs (Fig. 18). Interestingly, PSMCs did not respond to LPS stimulation (Fig. 18A), which could play a protective role preventing the inflammatory response to LPS inhalation. Additionally, PSMCs did not release IL-1β after IL-1α stimulation, as occurs in HASMCs (Fig. 18B). Therefore, this data could indicate that HASMCs could share mechanisms with PSMCs aimed at blocking IL-1β release in physiological conditions.

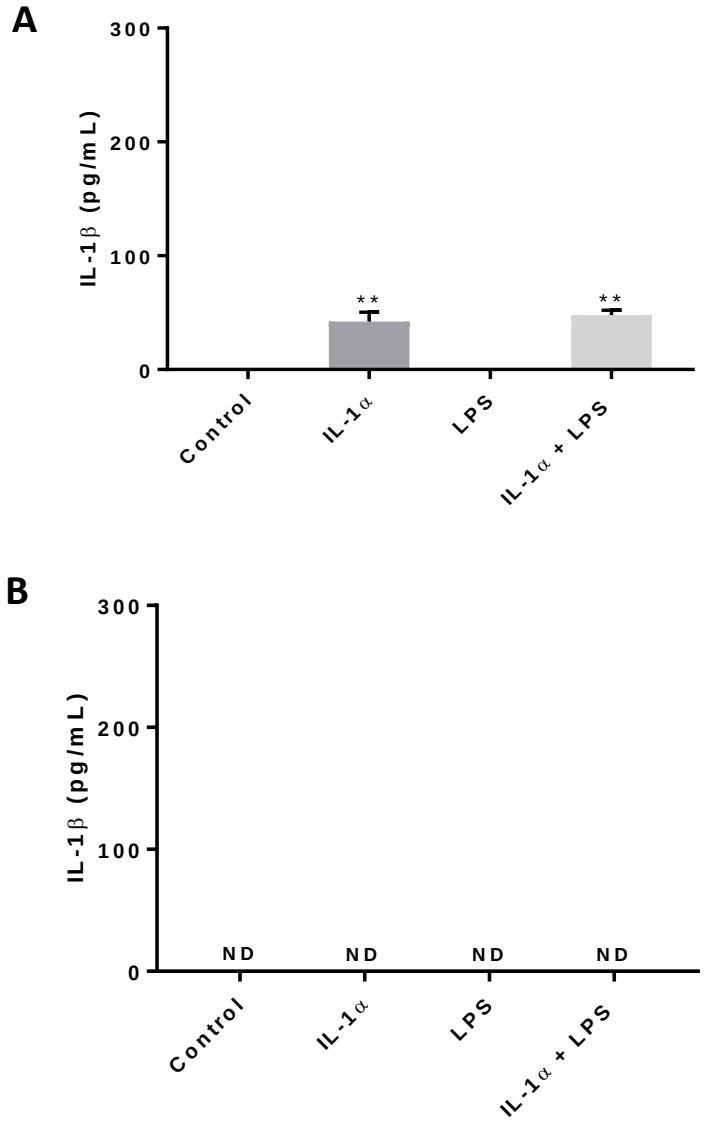


Fig. 18. PSMCs synthesise and process IL-1β in response to IL-1R1 ligation. (A,B) ELISA data indicating the level of mature IL-1β inside **(A)** or in the CM **(B)** of PSMCs treated ±IL-1α and/or LPS (7 h), as indicated. Data represents mean ± SEM, n = 3; p = ns > 0.05, * ≤0.05, **≤ 0.01, ***≤0.001

Discussion

VSMCs are the most abundant type of cell in the vasculature and play a critical role in the regulation of blood pressure and vessel remodelling[139-141]. Interestingly, VSMCs have also been identified as a source of diverse cytokines, chemokines and growth factors that can contribute to the atherosclerosis process[16, 142-144]. The effect of these proteins includes activation of endothelial cells; recruitment, activation and proliferation of leukocytes; proliferation and migration of SMCs; and stimulation of cytokine synthesis and release by endothelial cells, leukocytes and VSMCs (e.g. via paracrine/autocrine signalling)[142-144].

IL-1β is one of the proinflammatory cytokines that can be expressed by VSMCs in response to a varied set of stimuli (see above). Initial studies concluded that VSMCs synthesise pro-IL-1β, but serpin B9 blocks the activity of caspase-1 and thus the subsequent generation and secretion of mature IL-1β[118, 119]. Additionally, another study affirmed that ligation of CD40 in VSMCs triggers a different response and leads to processing and release of IL-1β into the extracellular milieu[120]. However, the results of this study remains controversial.

Intriguingly, the data that we report are not consistent with the findings described in these previous investigations. After IL-1α and/or LPS treatment, HASMCs contain mature IL-1β, which increases in a time-dependent manner. However, HASMCs treated with IL-1α and/or LPS or common NLRP3 inflammasome activators such as ATP, MSU crystals and nigericin do not release mature IL-1β into the conditioned media, and instead the cytokine accumulates inside the cell. Similar results were reported in VSMCs isolated from pulmonary arteries treated with IL-1α. Secretion of IL-1β via microvesicles, lysosomes, or exosomes that could not be detected by ELISA was ruled out.

The reason of the discrepancies between the literature and our data could be explained by use of cells isolated from different tissues. Whilst the studies of Schönbeck and Libby were performed in VSMCs from saphenous vein, I studied VSMCs extracted from aortas, and previous studies have showed differences in some phenotypic characteristics and cellular processes such as migration, cell growth and synthesis of extracellular matrix components in venous VSMCs compared to arterial VSMCs[145].

Furthermore, another possible reason for the disparity of the results is the methodology used in the studies. The group led by Schönbeck used coincubation of recombinant proteins (pro-IL-1β and caspase-1) and Western blot analysis and the data obtained was extrapolated to explain what occurs in the cells, whereas I studied conditioned media and cell lysates mainly by ELISA, a more sensitive technique that can generate more quantitative data and unveil

other aspects not observed previously. Likewise, I carried out experiments that were not included in the first investigations of Schönbeck and Libby, like determination of IL-1β by Western blotting in stimulated HASMC lysates, which successfully identified the bands corresponding to pro-IL-1β and mature IL-1β.

Concerning the results that I report, the activation of IL-1β in HASMCs is different compared to macrophages. In activated macrophages, IL-1β is synthesised as a proform, processed and subsequently secreted to exert its functions[132]. This is a two-step process, with the priming step (usually stimulation by a TLR ligand) inducing expression of pro-IL-1β and inflammasome components (e.g. NLRP3), and a second stimulus triggering assembly and activation of the inflammasome, which results in caspase-1 activation and cleavage and release of IL-1β[132]. However, HASMCs effectively only need an initial stimulation via IL-1R1 or TLR4 to produce mature IL-1β. In addition, common NLRP3 inflammasome activators that cause maturation and secretion of IL-1β in macrophages have no effect in HASMCs, suggesting that processing of IL-1β is likely NLRP3 inflammasome-independent.

Interestingly, this phenomenon of IL-1β activation after one stimulus has been previously described in monocytes. Dinarello and collaborators demonstrated that TLR ligation only triggers production, cleavage and secretion of IL-1β in monocytes, which was suggested to due to constitutive expression of active caspase-1[146].

On the other side, dendritic cells are immune cells that respond in a different manner. Constitutively, dendritic cells do not express IL-1β, but when treated with LPS or TNF produce and secrete biological inactive pro-IL1β[147]. By contrast, coculture of dendritic cells with activated CD4+ T cells leads to expression of the proform without release of either pro-IL-1β or of mature IL-1β into the conditioned media[147]. Additionally, dendritic cells upon CD8+ T cells stimulation process and secrete mature IL-1β[147]. However, the phenomena that I describe in VSMCs by which pro-IL-1β is cleaved and accumulates inside the cell, instead of being released into the extracellular milieu, is completely unexpected and has not been previously identified in any other cell type.

The possible causes for the failure of IL-1β to exit VSMCs are multiple. A potential explanation is the misfolding of IL-1β. IL-1β belongs to the β-trefoil family and the process of formation of the definitive protein structure is slow and complex due to the occurrence of folding/unfolding and refolding events (backtracking)[148, 149]. The alteration of this physiological process due to modifications of the intracellular conditions (e.g. pH, ionic strength, pressure) can cause changes of the final three-dimensional structure of the protein followed by alterations of the activity and release of IL-1β by VSMCs[150].

Another hypothesis is the existence of post-translational modifications that affect the capacity of IL-1β to be released from VSMCs. Post-translational modifications can modulate the conformation, function and stability of proteins and have been described as a regulatory mechanism of the immune system[151].

Likewise, IL-1β cleavage in VSMCs could be caspase-independent and thus generate mature IL-1β with a different structure and properties compared to immune cell- derived IL-1β, causing the unusual persistence of the cytokine inside the cell. The experiments to explore this hypothesis will be explained in detail in the next chapter.

In conclusion, VSMCs have an atypical mechanism that governs processing and prevents the secretion of IL-1β, which has not been described previously in the literature. In fact, if mature IL-1β accumulates inside VSMCs *in vivo*, this could indicate that necrosis of VSMCs is even more pro-inflammatory than previously appreciated. Therefore, to characterise this novel mechanism is important as it expands our understanding of VSMC biology in physiological conditions, as opens the possibility that a potential protective cellular process could be perturbed and contribute to vascular inflammation and atherogenesis.

Summary:

- IL-1β is synthesised and activated by HASMCs after treatment with IL-1α and/or LPS.
- A single stimulus is enough to trigger IL-1β cleavage by HASMCs.
- IL-1β is not released from HASMCs after treatment with IL-1α, LPS or common NLRP3 inflammasome activators, instead IL-1β remains in the intracellular compartment.

4. Serine proteases cleave IL-1β in Vascular Smooth Muscle Cells

Introduction

IL-1β is a potent cytokine that promotes inflammation and is involved in a wide range of immune responses[152]. Because of this, the generation and activation of IL-1β are processes under tight regulation in order to prevent tissue injury[152]. In immune cells, the processing of IL-1β is classically mediated by the formation of the NLRP3 inflammasome complex, followed by caspase-1 activation, cleavage of the inactive pro-IL-1β into mature IL-1β and release into the extracellular compartment where it exerts systemic and local effects[153].

Conversely, VSMCs synthesise and cleave pro-IL-1β, but the resulting mature IL-1β is not secreted by the cells, according to our data shown in chapter 3. However, a limited number of previous studies have determined the mechanisms implicated in IL-1β activation in VSMCs. Initially, Schönbeck and collaborators concluded that although VSMCs express pro-IL-1β, they do not process it due to the presence of Serpin B9 that blocks caspase-1 activity[118, 119]. Nevertheless, these findings are not consistent with our observations described in chapter 3. In contrast, a more recent investigation performed by the group of Zhong has affirmed that VSMCs cultured in media containing sodium pyruvate and β-glycerophosphate undergo calcification, which leads to upregulation of the inflammasome components NLRP3, ASC and caspase-1, followed by inflammasome activation and release of IL-1β[121]. Similarly, the group led by Kim found that the protein high mobility group box 1 (HMGB1) binds to TLR2, TLR4 and RAGE receptors on VSMCs and causes increased expression of NLRP3, ASC and caspase-1, inflammasome activation and finally caspase-1 processing of IL-1β[122].

Saito and collaborators also reported that VSMCs produce and cleave IL-1β in response to lysosomal membrane permeabilisation via the NLRP3 inflammasome and caspase-1. In this study VSMCs were treated with Leu-Leu-O-methyl ester (LLME) that produces lysosomal membrane rupture and release of intralysosomal components into the cytoplasm. This process led to activation of NF-κβ (presumably through cathepsin B activity), upregulation of pro-IL-1β and NLRP3, and without requiring a second signal, activation of the NLRP3 inflammasome and caspase-1, and cleavage of pro-IL-1β into the active form[123]. In addition, Proudfoot and collaborators found that calcium phosphate particles induced IL-1β processing in VSMCs through phosphorylation of spleen tyrosin kinase (SYK) and activation of caspase-1[125].

Interestingly, our preliminary observations suggest that the generation of mature IL-1β in HASMCs does not require NLRP3 inflammasome activation. To confirm these findings and to determine the proteases involved in the cleavage of pro-IL-1β in VSMCs is crucial for various reasons; firstly, this work may unveil a potential IL-1β processing pathway that has not been reported previously, which would improve our understanding of the physiology of VSMCs.

Second, finding an explanation for the persistence of mature IL-1β inside VSMCs may reveal new biology. For example, if pro-IL-1β is processed by a non-caspase protease at an alternative site, perhaps the variant amino acid sequences and/or new secondary/tertiary structure of mature IL-1β subsequently affects the ability of the protein to exit the cell. Finally, novel pathways specific to VSMCs open the potential for new drugs that limit the inflammation driven by VSMCs-derived IL-1β.

Therefore, in this chapter I will explore the role of key proteins previously implicated in the processing of IL-1β, including NF-κβ, NLRP3 inflammasome components and inflammatory caspases.

Aims:

1. To determine the role of NF-κβ signalling in the production of mature IL-1β by HASMCs.

2. To study if the NLRP3 inflammasome is implicated in IL-1β processing by HASMCs.

3. To investigate any role for caspases in the cleavage of IL-1β by HASMCs.

4.1 NF-κβ activation is required for generation of mature IL-1β.

NF-κβ is a well-known transcription factor that regulates the expression of numerous genes involved in division, differentiation and cell viability[154]. Likewise, NF-κβ plays an important role in immunity and inflammation by controlling the transcription of a varied set of proteins, such as chemokines, growth factors, cell adhesion molecules, cell surface receptors and cytokines, including pro-IL-1β[154-156]. Thus, I studied if activation of NF-κβ is implicated in the synthesis and/or cleavage of IL-1β in HASMCs. To this end, cells were incubated with isohelenin, a cell-permeable and irreversible inhibitor of NF-κβ, followed by IL-1α stimulation. After treatment, samples were analysed by ELISA to determine the concentrations of cleaved IL-1β inside HASMCs. The results showed a massive reduction in the level of mature IL-1β after NF-κβ inhibition (Fig. 19), which indicates that IL-1β synthesis and cleavage is dependent on NF-κβ activation, as expected.

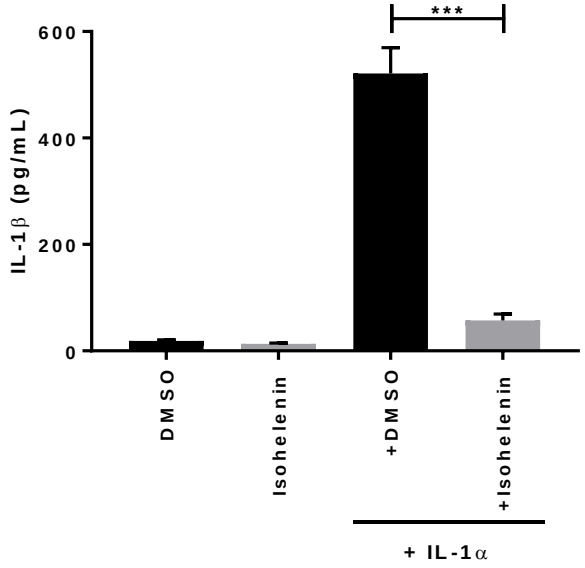


Fig. 19. Synthesis and cleavage of IL-1β in HASMCs is mediated by NF-κβ activation. ELISA data showing the level of mature IL-1β inside HASMCs treated with isohelenin (30 mins) and/or IL-1α (6 h), as indicated. Data represents mean ± SEM, n = 3; p = *** ≤0.001.

4.2 The NLRP3 inflammasome is not implicated in the processing of IL-1β in HASMCs.

Inflammasomes are multimeric complexes assembled in response to pathogens or endogenous signals that lead to caspase-1 activation and subsequent cleavage of IL-1β and IL-18[153]. NLRP3 is the best characterised inflammasome and has been implicated in activation of IL-1β in immune cells[153]. However, as described in chapter 3, common NLRP3 inflammasome inducers do not increase the generation of mature IL-1β in HASMCs, suggesting that IL-1β production is NLRP3-inflammasome independent. To confirm these observations, I incubated Thp1 cells and HASMCs with MCC950, a selective inhibitor that blocks ASC oligomerization and canonical and non-canonical NLRP3 inflammasome activation[157], followed by LPS/Nigericin or IL-1α treatment, respectively. The results showed that MCC950 successfully inhibits the production of mature IL-1β in Thp1 cells (Fig. 20A), but does not affect mature IL-1β generation in HASMCs (Fig. 20B). Thus, we conclude that NLRP3 inflammasome assembly is not required for the activation of IL-1β in HASMCs after IL-1α treatment.

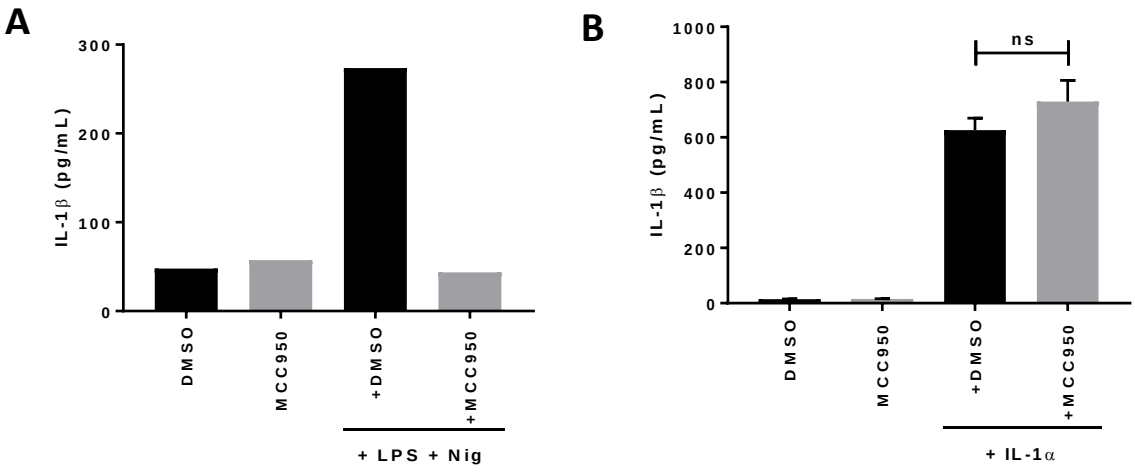


Fig. 20. Maturation of IL-1β in HASMCs is not dependent on NLRP3 inflammasome activation. (A,B) ELISA data showing the level of mature IL-1β in the CM of Thp1 cells treated with MCC950 (30 mins) and/or LPS (6 h)/nigericin (3 h) **(A)**, or the level of mature IL-1β inside HASMCs treated with MCC950 (30 mins) and/or IL-1α (6 h) **(B)**, as indicated. Data represents mean ± SEM, n = 1 (A), 3 (B); p = ns >0.05

4.3 Serpin B9 knockdown does not increase generation of mature IL-1β by HASMCs.

Schönbeck and collaborators determined that IL-1β is not processed in VSMCs due to Serpin B9 blocking the proteolytic activity of caspase-1[119]. Although I see IL-1β processing in HASMCs I knocked down *SERPINB9* using siRNA to see if this led to caspase-1 activation and subsequent increased IL-1β cleavage and release. However, *SERPINB9* knockdown did not increase the production of mature IL-1β compared to the non-targeting control (Fig. 21A). Likewise, I observed that *SERPINB9* silencing did not cause IL-1β release (Fig. 21B). Thus, I concluded that serpin B9 does not regulate maturation of IL-1β in HASMCs, in contrast to the data reported in the literature.

Interestingly, I observed an increment in the concentration of mature IL-1β inside HASMCs treated with non-targeting siRNA compared to the control (Fig. 21A). As VSMCs express TLR3 receptors[13] that can sense double stranded RNA such as siRNA, leading to IL-1β processing in other cell types[158-160], I studied the effect of siRNA incubation on IL-1β maturation in HASMCs. Nevertheless, this showed that the increased mature IL-1β was not produced by siRNA treatment, but rather as a consequence of Optimem-Lipofectamine RNAiMAX addition alone (Fig. 22). The underlying reason for these findings are not clear, however a possible explanation of this phenomenon could be the presence of LPS in the Optimem or RNAimax which induced synthesis of IL-1β by HASMCs.

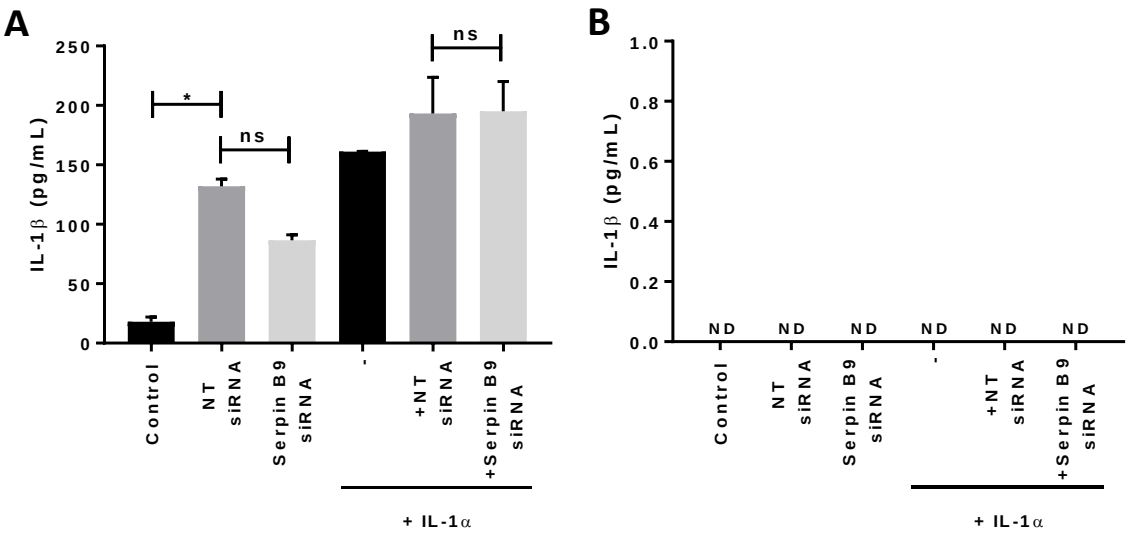


Fig. 21. Serpin B9 does not regulate IL-1β processing in HASMCs. (A,B) ELISA data showing the level of mature IL-1β inside **(A)** and in the CM **(B)** of HASMCs treated with non-targeting or *SERPINB9*-targeting siRNA (48 h), ±IL-1α (7 h), as indicated. Data represents mean ± SEM, n = 3; p = ns >0.05, * ≤0.05.

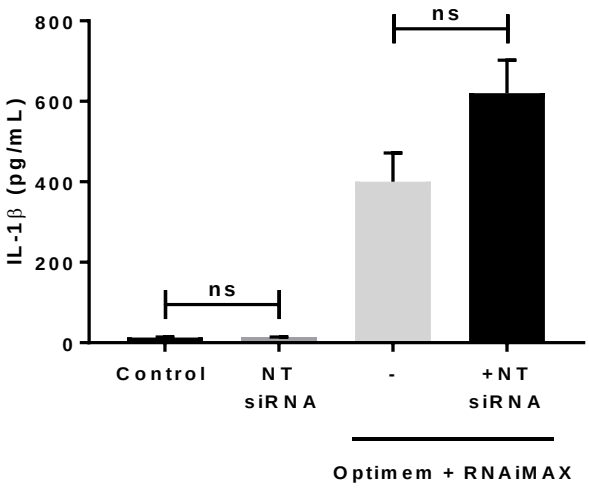


Fig. 22. Non-targeting siRNA does not increase IL-1β concentration inside HASMCs. ELISA data showing the level of mature IL-1β inside HASMCs treated with non-targeting siRNA and/or Optimem/RNAiMax (48 h), as indicated. Data represents mean ± SEM, n = 2; p = ns >0.05.

4.4 IL-1β from activated HASMCs and Thp1 cells exhibits the same banding pattern on a Western blot.

Caspases are a family of cysteine proteases that catalyse hydrolysis of aspartic acid peptide bonds[161]. Canonical (caspase-1) and non-canonical inflammatory caspases (caspase-4, -5, -8) are implicated in the processing of IL-1β in multiple cell types, including Thp1 cells[18, 82, 87]. I wondered whether caspases cleave IL-1β in HASMCs. To study this, we prepared lysates of stimulated HASMCs and Thp1 cells and performed Western blotting. The results showed that pro-IL-β (p31), intermediate forms and mature IL-1β (p17) from Thp1 cells and HASMCs migrated to the same positions on a Western blot (Fig. 23). This indicates that IL-1β cleavage is probably mediated by the same proteases and that it is likely a caspase.

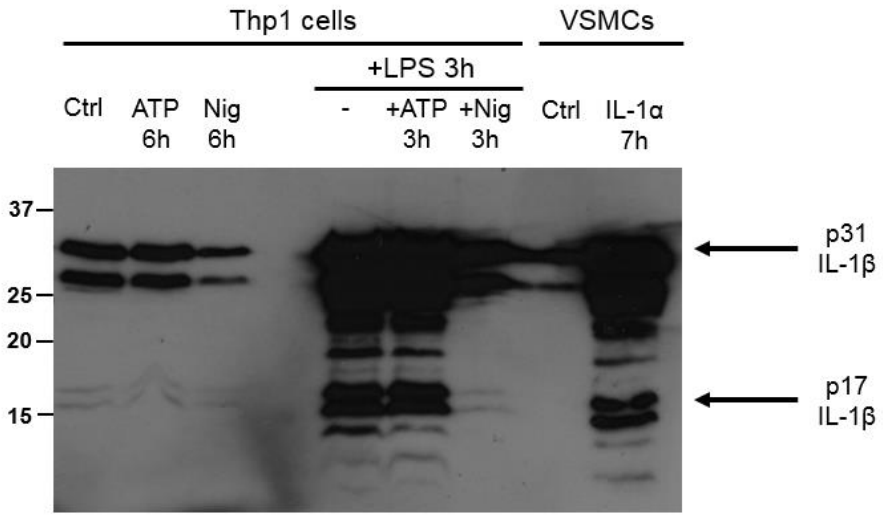


Fig. 23. IL-1β in activated HASMCs and Thp1 cells displays the same cleavage pattern. Western blot for IL-1β in Thp1 and HASMC lysates after treatment with LPS, ATP, nigericin and/or IL-1α, as indicated. Representative of n = 2.

4.5 HASMCs express caspase-1, -4 and -8.

According to the previous data, IL-1β maturation in VSMCs is likely caspase-dependent. Thus, I investigated gene expression of canonical and no canonical caspases using qPCR. This showed that HASMCs express *CASP1*, *CASP4* and *CASP8*, but not *CASP5* (Fig. 24). Additionally, IL-1α treatment did not grossly change expression of these protease genes.

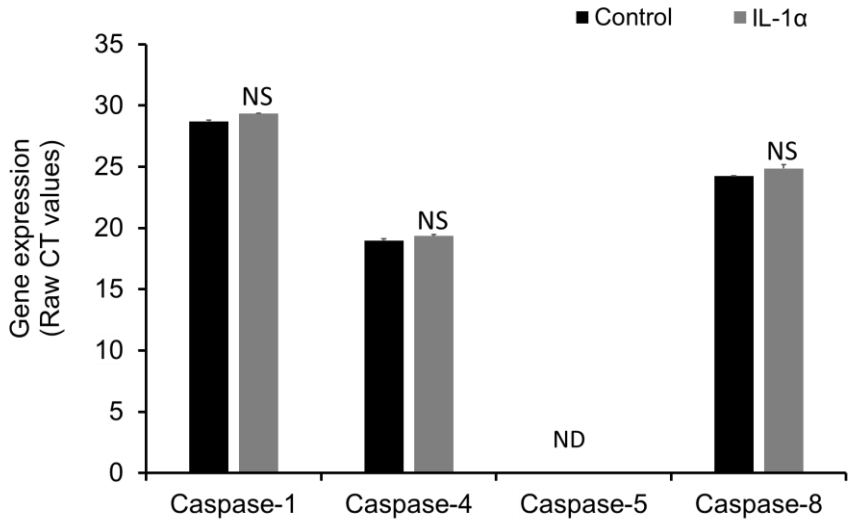


Fig. 24. HASMCs express caspase-1, -4 and -8, but not caspase-5. qPCR data showing expression of caspases in HASMCs ±IL-1α treatment (7 h), as indicated. ND = not detected. Data represent mean ± SEM. n = 2. p = ns >0.05.

4.6 Caspase inhibitors do not block mature IL-1β production in HASMCs.

The activity of caspases can be blocked by interaction with specific inhibitors[162]. Therefore, I used pan-caspase and selective caspase inhibitors to study the role of caspases in the cleavage of IL-1β in HASMCs. Initially, I tested the effectiveness of ZVAD, a well-known pan-caspase inhibitor[163], and QVD, a purported less toxic general caspase inhibitor[164] in Thp1 cells. This showed that both inhibitors significantly decreased the production of mature IL-1β (Fig. 25A,B), with the reduction in pro-IL-1β generation estimated to be 66% (Fig. 25C,D).

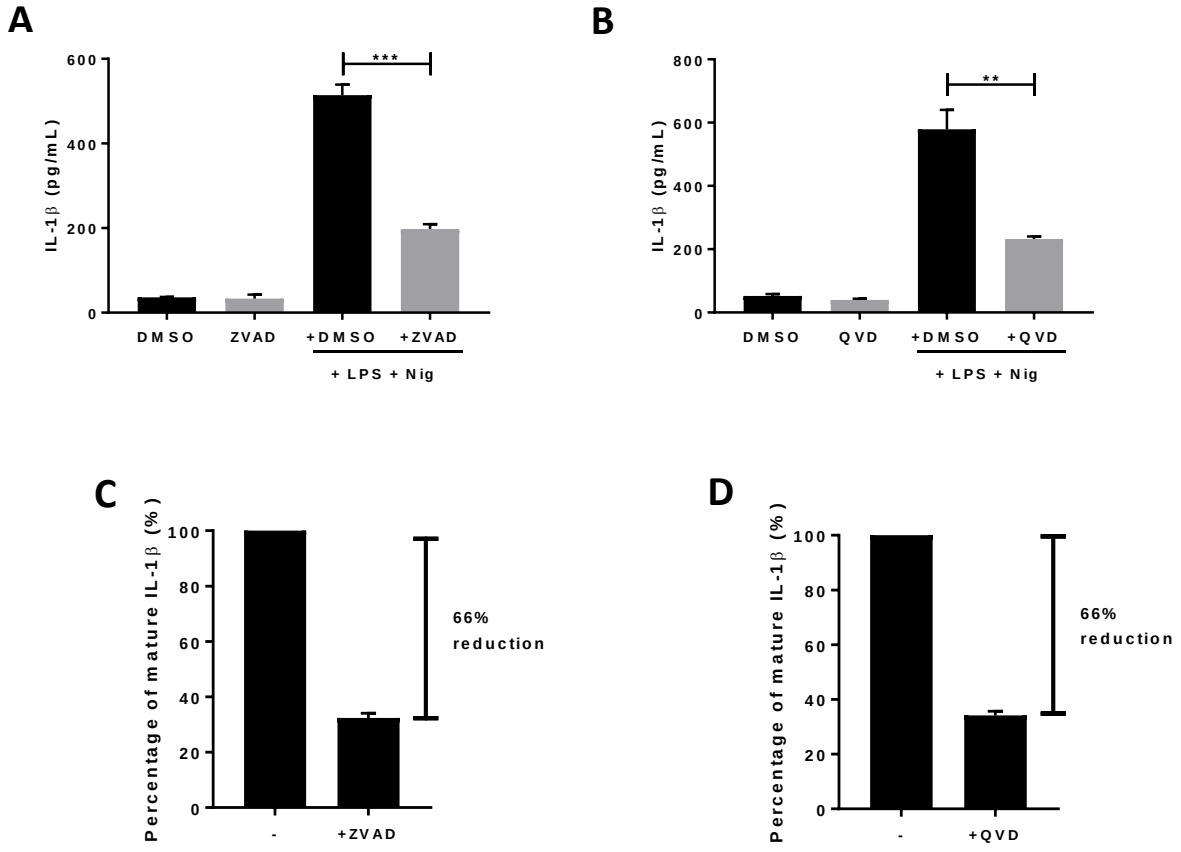


Fig. 25. Pan-caspase inhibitors decrease mature IL-1β production by Thp1 cells. (A,D) ELISA data showing the level of IL-1β in the CM of Thp1 cells treated with ZVAD **(A)** or QVD **(B)** (30 mins) before LPS (3 h)/nigericin (6 h), as indicated. **(C,D)** Percentage of mature IL-1β production by Thp1 cells treated ± ZVAD **(C)** or ± QVD **(D)**. Data represent mean ± SEM. n = 2 (A), 3 (B); p = ** ≤0.01, *** ≤0.001.

However, when I performed similar experiments in HASMCs, ZVAD and QVD did not cause any reduction in the generation of p17 IL-1β (Fig. 26A,B). Nevertheless, it is important to mention that these observations are only based on ELISA.

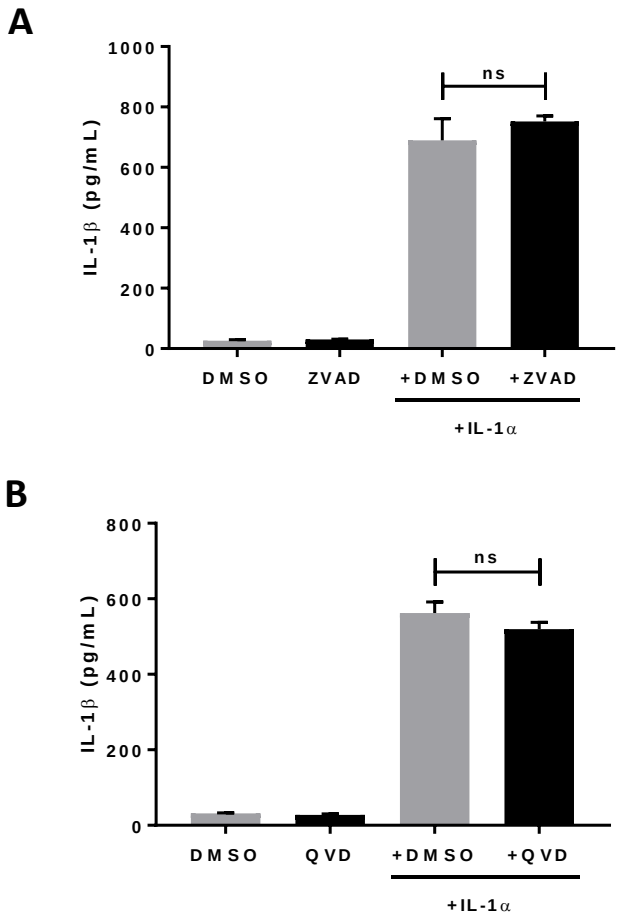


Fig. 26. Pan-caspase inhibitors do not reduce mature IL-1β generation in HASMCs. (A,B) ELISA data showing the level of IL-1β inside VSMCs after treatment with ZVAD **(A)** or QVD **(B)** (30 mins) before IL-1α (6 h), as indicated. Data represent mean ± SEM. n = 3; p = ns >0.05.

Therefore, titration experiments using ZVAD were carried out to investigate if higher concentrations of this compound are required to block IL-1β production in HASMCs. The results in Thp1 cells showed a progressive reduction in the generation of mature IL-1β with increasing ZVAD concentration (Fig. 27A). In contrast, no clear relationship between concentration of ZVAD and production of mature IL-1β was detected in HASMCs (Fig. 27B), confirming ZVAD could not prevent the generation of mature IL-1β in HASMCs.

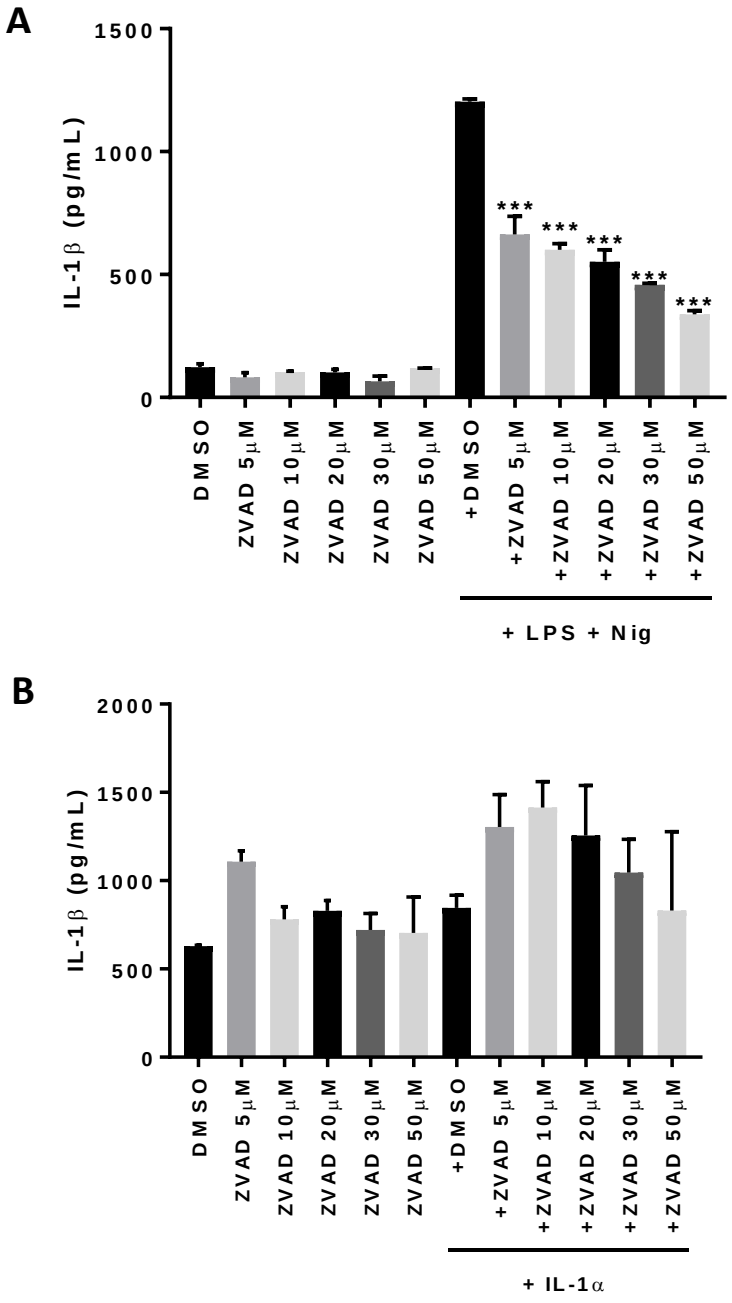


Fig. 27. Higher ZVAD concentrations do not prevent generation of mature IL-1β in HASMCs. (A,B) ELISA data showing the level of mature IL-1β in the CM of Thp1 cells treated with ZVAD (30 mins) before LPS (6 h)/nigericin (3 h) **(A)**, and the level of mature IL-1β inside HASMCs incubated with ZVAD (30 mins) and/or IL-1α (6 h) **(B)**, as indicated. Data represent mean ± SEM. n =2; p = *** ≤0.001.

As a consequence of the lack of effect of caspase inhibitors, I wondered whether ZVAD and QVD cannot enter HASMCs or are quickly pumped out of the cells before blocking caspase activity. To corroborate this, HASMCs were preincubated with QVD, and then apoptosis induced using the Fas-ligating antibody CH11 and cycloheximide. Interestingly, QVD could effectively prevent cell death (Fig. 28), demonstrating that this inhibitor indeed enters HASMCs and inhibits apoptotic caspase activation, as expected.

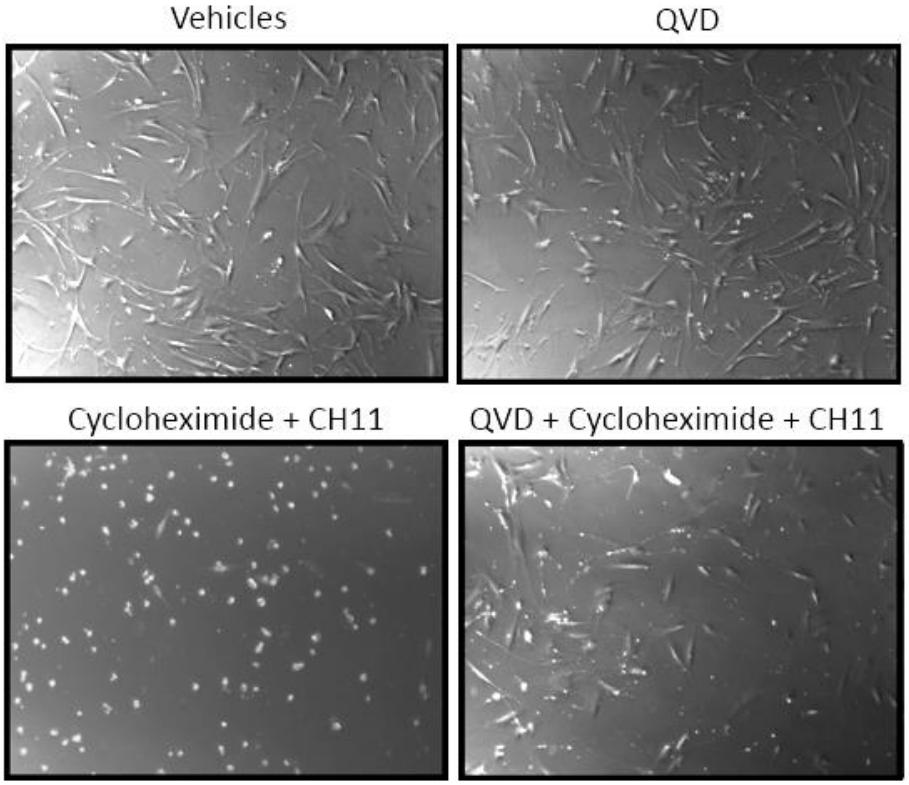


Fig. 28. QVD prevents cell death of HASMCs incubated with cycloheximide and CH11. Microscopic phase contrast images of HASMCs treated with QVD (30 mins) before cycloheximide/CH11 (24 h), as indicated.

Next, I studied the effect of more selective caspase inhibitors on IL-1β cleavage. I used YVAD and the non-peptide VX-765, both inhibitors of caspase-1; LEVD, inhibitor of caspase-4; and IETD, inhibitor of caspase-8. However, the results were similar with significant reduction in p17 IL-1β generation in Thp1 cells (Fig. 29A-D) but no reduction in HASMCs (Fig. 29E-H). Hence, based on these findings, I finally conclude that IL-1β maturation in HASMCs is not mediated by caspase activation. This also reveals that supposedly caspase-specific inhibitors show considerable promiscuity toward other caspase family members.

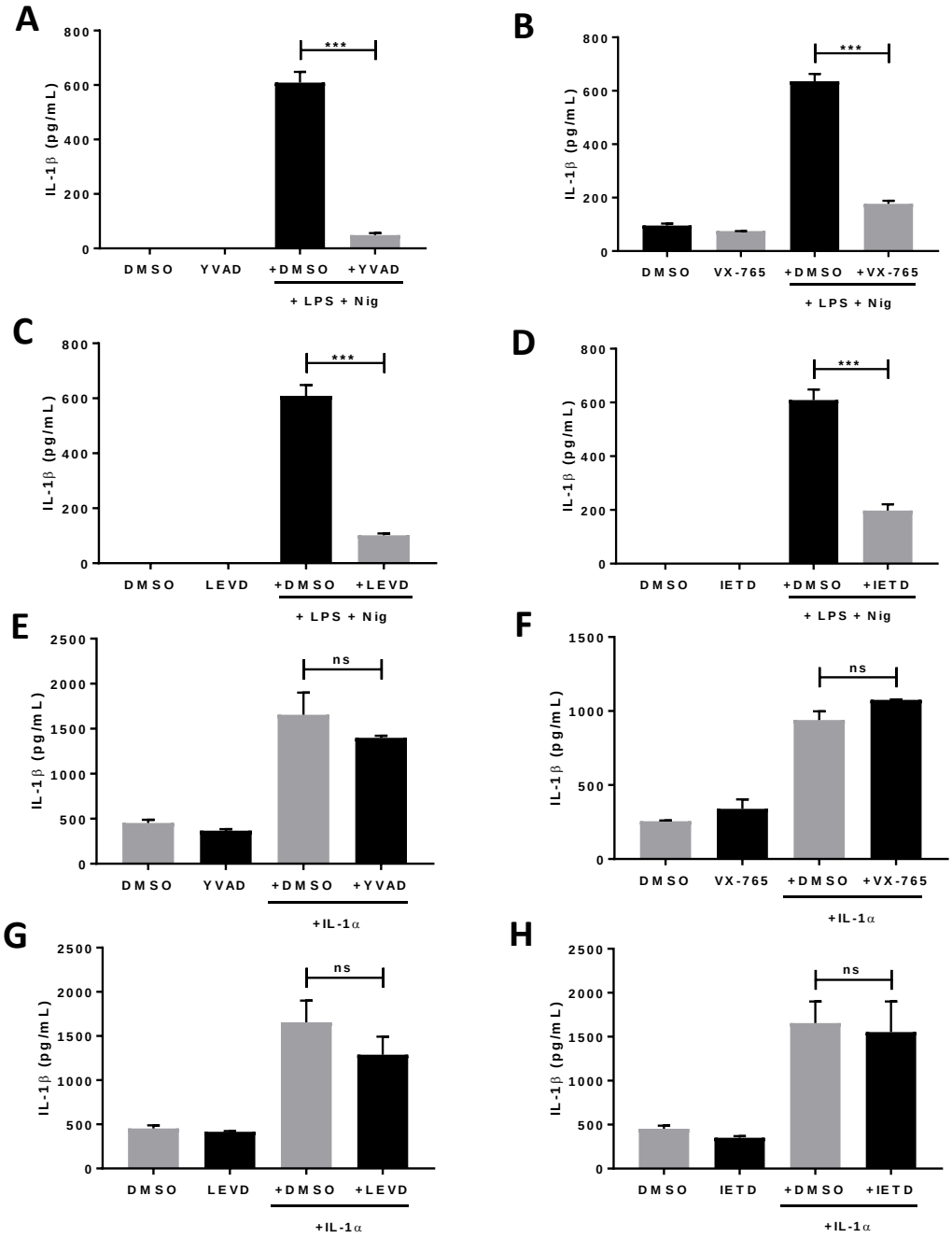


Fig 29. Selective caspase inhibitors do not reduce mature IL-1β generation in HASMCs. **(A-H)** ELISA data showing the level of mature IL-1β in the CM of Thp1 cells treated with LPS (6 h)/nigericin (3 h) **(A-D)** or inside HASMCs treated ± IL-1α (6 h) **(E-H)**, ± YVAD, VX-765, LEVD, IETD (30 mins) before stimulation, as indicated. Data represent mean ± SEM. n = 3; p = ns >0.05, *** ≤0.001.

4.7 Cathepsin G is likely responsible for cleavage of pro-IL-1β in HASMCs.

Pro-IL-1β is typically processed by caspase-1. Nevertheless, previous investigations have shown that other proteases also induce IL-1β activation. The results of coincubation of recombinant proteins and proteases have shown that the serine protease elastase cleaves pro-IL-1β[92, 93]. Additionally, a recent study proposed that neutrophil elastase, potentially secreted by monocytes, is internalised by endothelial cells and induces IL-1β maturation and release to the extracellular compartment[165]. As VSMCs express elastase[166], I studied if IL-1β cleavage in this cell type is mediated by this. To this end, I used BAY 678, a selective and cell-permeable inhibitor of elastase, followed by IL-1α treatment. The results showed that the generation of mature IL-1β is not blocked after elastase inhibition (Fig. 30), which indicates that elastase does not process pro-IL-1β to mature IL-1β in HASMCs.

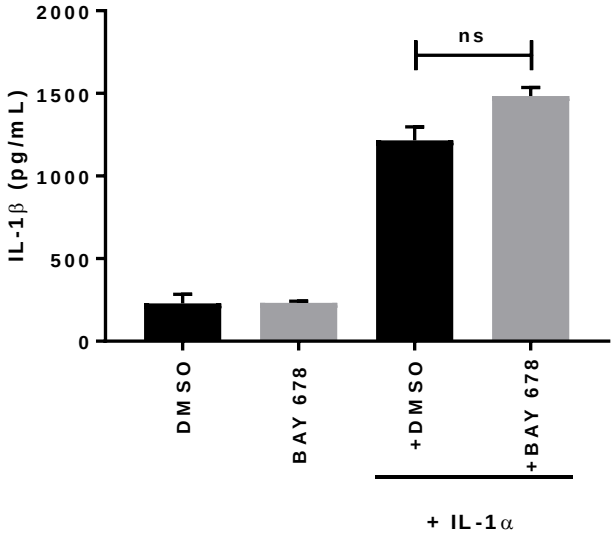


Fig. 30. Elastase does not cleave IL-1β in HASMCs. ELISA data showing the level of mature IL-1β inside HASMCs after treatment with BAY 678 (30 mins) before IL-1α for (6 h), as indicated. Data represent mean ± SEM. n = 3; p = ns >0.05

Similarly, granzyme B is a serine protease that induces apoptosis and is mostly express by natural killer and cytotoxic T cells[167]. Nevertheless, other immune and non-immune cell types can also express granzyme B, including vascular smooth muscle cells in advanced atherosclerosis lesions and aortic aneurysms[168, 169]. Granzyme B does not cleave IL-1β in coincubation assays of recombinant proteins[167]. However, as serpin B9 can inhibit granzyme B[170] I wondered if serpin B9 prevents IL-1β cleavage in VSMCs, as shown by Schönbeck[119], but through inhibition of granzyme B activity instead of caspase-1. To assess this hypothesis, I treated HASMCs with ZAAD, a selective, irreversible and cell-permeable granzyme B inhibitor, followed by IL-1α stimulation. This showed that ZAAD does not decrease mature IL-1β generation (Fig. 31), confirming that granzyme B does not cleave IL-1β in HASMCs.

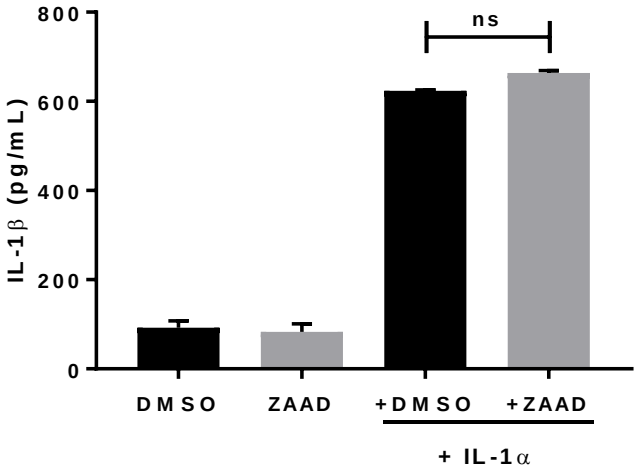


Fig. 31. Granzyme B does not cleave IL-1β in HASMCs. ELISA data showing the level of mature IL-1β inside HASMCs after treatment with ZAAD (30 mins) before IL-1α (6 h), as indicated. Data represent mean ± SEM. n = 3; p = ns >0.05.

Some matrix metalloproteinases (MMPs) have also been implicated in IL-1β maturation. Libby and collaborators used cell free systems to determine that IL-1β is cleaved by MMP-2, MMP-3 and MMP-9, and that the C-terminal products generated possess biological activity, which was proved in D10.G4.1 cell proliferation assays[91]. In addition, a recent investigation showed that activated eosinophils express IL-1β, and the cleavage and subsequent release of this cytokine is dependent on MMP-9 activity[171]. Multiple MMPs, including MMP-2, -3 and -9, are highly expressed in atherosclerotic plaques[172]. Interestingly, VSMCs only synthesise MMP-2 under basal conditions[173], but diverse stimuli such as mechanical injury, proinflammatory cytokines (IL-1, IL-4, TNF), growth factors, high glucose and LPS cause upregulation of MMP-1, -2, -3, -9[173-177]. Although MMPs are classically synthesised as proforms, released to the extracellular compartment and then activated to mediate degradation of extracellular matrix proteins, some studies have found active MMP-2 in the intracellular space of multiple cell types, where they cleave diverse substrates under physiological and pathological conditions[178-181]. Likewise, MMP-3, MMP-7, MMP-9, MMP-10, MMP-14 and MMP-26 can exert proteolytical activities intracellularly[182]. Hence, I evaluated the effect of the general metalloproteinase inhibitor GM-6001, and the selective MMP-2 Inhibitor I on mature IL-1β generation in HASMCs after IL-1α treatment. The data showed that GM-6001 and MMP-2 Inhibitor I do not reduce the levels of cleaved IL-1β after IL-1α treatment (Fig. 32). Therefore, I conclude that metalloproteinases are not responsible for the processing of IL-1β in HASMCs.

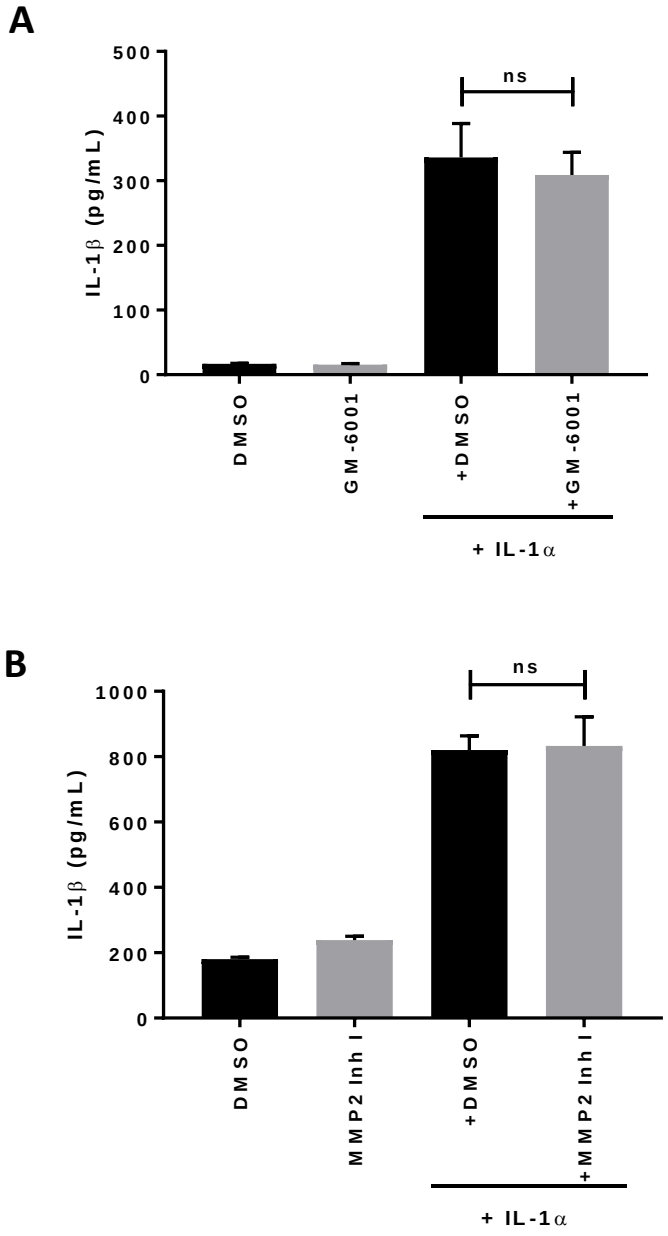


Fig. 32. Metalloproteinases do not process IL-1β in HASMCs. (A,B) ELISA data showing the level of mature IL-1β inside HASMCs after treatment with GM-6001 **(A)**, MMP-2 Inhibitor I **(B)** (30 mins) before IL-1α (6 h), as indicated. Data represent mean ± SEM. n = 3; p = ns >0.05.

Cathepsins are a family of serine, cysteine and aspartyl proteases that play a role in diverse biological processes including cell division, apoptosis, autophagy, proteolysis, immunity and coagulation[183]. The group of cysteine cathepsins includes cathepsins B, C, F, H, K, L, O, S, V, W and Z[183]. In microglial cells, cathepsin B actives caspase-1 leading to IL-1β processing[184]. Likewise, Rock and collaborators postulated that cathepsin C can mediate IL-1β cleavage through activation of neutrophil serine proteases (e.g. elastase, cathepsin G, and protease 3)[185]. In addition, previous investigations have shown that VSMCs do not express cathepsin S in physiological conditions, but cathepsin S and K are synthesised in atherosclerotic plaques[186]. Cathepsin S can also be induced in response to IL-1β, TNF-α and IFN-γ treatment[187] and localises to the cell surface where it contributes to cell migration through matrix degradation[188]. Finally, cathepsin B, L, X also promote NLRP3 inflammasome activation[189]. Given this context, I studied the role of cysteine proteases in mature IL-1β production by using E64, a cell permeable and irreversible cysteine protease inhibitor. The results showed no inhibition of mature IL-1β generation after E64 treatment (Fig. 33), ruling out the involvement of cysteine cathepsins in the activation of IL-1β in HASMCs.

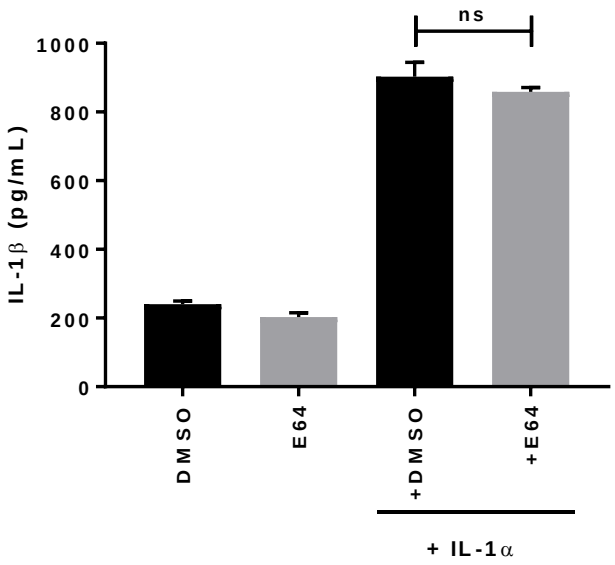


Fig. 33. Cysteine cathepsins do not cleave IL-1β in HASMCs. (A,B) ELISA data showing the level of mature IL-1β inside HASMCs after treatment with E64 (30 mins) before IL-1α (6 h), as indicated. Data represent mean ± SEM. n = 3; p = ns >0.05.

Another member of the cathepsin family that has been implicated in the processing of IL-1β is the serine protease cathepsin G that cleaves pro-IL-1β in coincubation assays with recombinant proteins[92, 93]. Interestingly, cathepsin G is barely expressed in normal arteries, but is upregulated in VSMCs of atherosclerotic lesions and abdominal aortic aneurysms, as well

as in VSMCs incubated with cytokines such as IFNγ, TNFα, IL-6, FGF-2 or maintained in high glucose medium[88, 190, 191]. Likewise, the serine proteases granzyme A and proteinase 3 also process IL-1β[89, 192, 193]. Thus, HASMCs were incubated with dichloroisocoumarin, an irreversible and non-selective inhibitor of serine proteases that acts on granzyme A, B, H, cathepsin G, neutrophil elastase and proteinase 3. Importantly, the results showed that HASMCs treated with dichloroisocoumarin have a significant, dose-dependent reduction in the level of mature IL-1β compared to control (Fig. 34), which indicates that any of the proteases mentioned above could be responsible for IL-1β processing in HASMCs.

However, the role of elastase and granzyme B was ruled out by the use of the selective inhibitors BAY 678 and ZAAD, respectively (see above; Fig. 30, 31). Proteinase 3, granzyme A and H are expressed in immune cells[194, 195], and no investigations have demonstrated the synthesis of these proteases by VSMCs. Taken together this suggests that IL-1β may be activated by cathepsin G. However, additional experiments need to be performed in order to confirm these findings and exclude off-target effects of dicloroisocumarin on the IL-1β pathway.

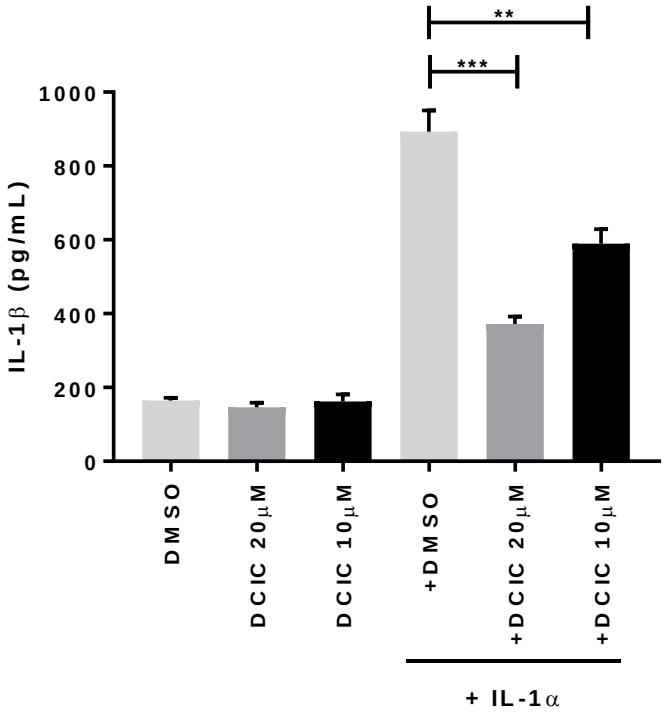


Fig. 34. Generation of mature IL-1β in HASMCs is prevented by dichloroisocoumarin. ELISA data showing the level of mature IL-1β inside HASMCs after treatment with dichloroisocoumarin -DCIC- (30 min) before IL-1α (6 h), as indicated. Data represent mean ± SEM. n = 3; p = ** ≤0.01, *** ≤0.001.

Discussion

IL-1β is synthesised as an inactive precursor that requires cleavage to produce a biological effect[196]. Thus, processing of IL-1β is an important regulator of IL-1β activity[196]. In immune cells, the mechanisms that lead to IL-1β cleavage have been widely studied and characterised[196]. Nevertheless, few studies have examined the processing of IL-1β in VSMCs. In these investigations diverse agents such as β-glycerophosphate, high mobility group box 1, Leu-Leu-O-methyl ester and calcium phosphate particles were utilised to stimulate VSMCs, and the results indicated that the generation of mature IL-1β is dependent on NLRP3 inflammasome assembly and/or caspase-1 activation[121-123, 125].

Our data demonstrated that the NF-κβ signalling pathway mediates IL-1β synthesis and cleavage in HASMCs after IL-1R1 ligation. However, NLRP3 inflammasome activation is not involved in the cleavage of IL-1β. Additionally, although HASMCs express caspase-1, -4 and -8, none of these proteases play a role in the processing of IL-1β. These conclusions were based on the results obtained after using multiple general and selective caspase inhibitors. I also confirmed that serpin B9 does not regulate IL-1β cleavage. Similarly, other enzymes such as elastase, granzyme B, matrix metalloproteinases and cysteine cathepsins are not implicated in IL-β activation in HASMCs. However, from the data I have, I believe Cathepsin G is the most likely mediator of IL-1β cleavage in HASMCs.

These findings show that HASMCs and macrophages both contain distinct mechanisms to generate active IL-1β. In both cell types, activation of the NF-κβ signalling pathway is required to express pro-IL-1β. However, the processes leading to cleavage of pro-IL-1β is different in HASMCs compared to the classical pathway described for macrophages. Macrophages require the NLRP3 inflammasome to activate caspase-1 for IL-1β cleavage, while our data indicates HASMCs do not use NLRP3 or caspase-1. Similarly, other inflammasomes, such as NLRP1, NLRP2, NLRC4 and AIM2, are also unlikely to be either implicated in IL-1β activation in HASMCs, because all these multimeric protein complexes, despite being composed of different sensor and adaptor proteins, all exhibit caspase-1 as a common downstream effector[196].

Concerning inflammasome-independent pathways, diverse cell types such as macrophages, neutrophils, NK, mast, and epithelial cells can utilise elastase, proteinase 3, cathepsins, granzyme A, chymase, chymotrypsin and metalloproteinases to process IL-1β[196]. Other studies have also described that certain microorganisms (e.g. *Candida albicans, Entamoeba histolytica, Staphylococcus aureus*) can secrete proteases that directly cleave pro-IL-1β into

mature IL-1β, contributing to the inflammatory response[196-198]. The cleavage sites within pro-IL-1β for these aforementioned proteases have been characterized (Fig. 35).

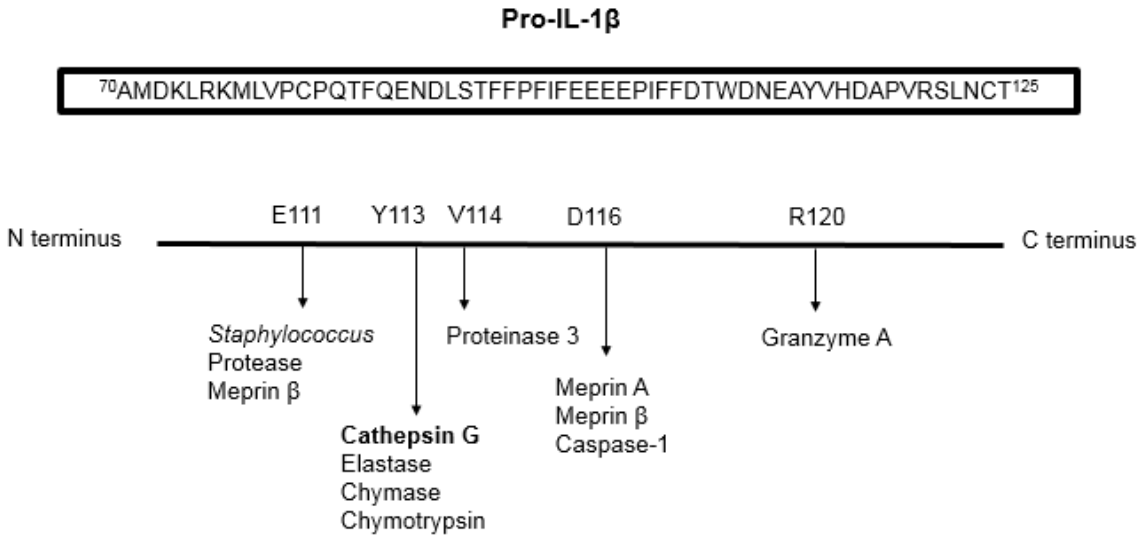


Fig. 35. Representation of the amino acid sequence and protease cleavage sites in pro-IL-1β. Adapted from *Netea, 2015.*

The data presented in this chapter suggest that cathepsin G is likely responsible for the activation of IL-1β in HASMCs. Cathepsin G is encoded by the *CTSG* located on chromosome 14 at q11.2, and is typically expressed by B cells, monocytes, microglia and dendritic cells[199, 200]. This protease is synthesised as a proform of 255 amino-acid residues, subsequently processed by cathepsin C and stored in intracellular azurophilic granules or secretory lysosomes[199, 201, 202]. Finally, a diverse set of stimuli (e.g. certain medications, phagocytosis, immune complexes) trigger the secretion of cathepsin G to the extracellular milieu where it exerts various biological effects, including increased vascular permeability, alteration of chemokines leading to improved chemoattraction, activation of pro-inflammatory receptors and cleavage of cytokines[199, 203, 204].

Nevertheless, additional studies are essential to corroborate the role of cathepsin G in IL-1β processing in HASMCs. Firstly, as VSMCs are reported to express negligible levels of cathepsin G under physiological conditions, I would investigate if cathepsin G gene expression changes in IL-1α-treated HASMCs. I would expect upregulation of *CTSG* in HASMCs treated with IL-1α or LPS. Likewise, it would be pertinent to investigate the level of IL-1β cleavage after silencing cathepsin G (e.g. with siRNA or antisense oligonucleotides), which would confirm the initial observation of reduced IL-1β cleavage after treating HASMCs with dichloroisocumarin. This is particularly relevant due to the contradictory results using E64, as these experiments

showed no reduction in HASMC IL-1β cleavage after blocking cathepsin C - the enzyme responsible for activation of multiple serine proteases, including cathepsin G[200, 201, 205].

Other important experiments to be performed in order to corroborate our hypothesis include demonstrating the ability of recombinant cathepsin G to cleave either recombinant pro-IL-1β or HASMC-derived pro-IL-1β in cell-free systems, to perform colocalization studies of cathepsin G and IL-1β, as well as to identify the cleavage site of HASMC-derived p17 IL-1β by the use of mass spectrometry. In the case of IL-1β activated by cathepsin G, pro-IL-1β is reported to be cleaved at Y113 (Fig. 35).

In addition, the cleavage of pro-IL-1β by a serine protease could be the cause for no secretion of IL-1β from HASMCs, for example due to modifications to the structure of mature IL-1β. However, there are other potential reasons including no expression of gasdermin D or the existence of an inhibitor in HASMCs that prevents pyroptosis and the subsequent release of IL-1β.

Interestingly, IL-1β localises mainly to the cytosol and in lower concentrations inside endolysosomal vesicles of monocytes treated with LPS[132]. In other cell types (e.g. mesangial cells, fibroblast and microglia), IL-1β can also be found in the nucleus, but its intranuclear actions are unknown[206]. In VSMCs, the subcellular localisation of IL-1β has not been reported previously, and to investigate this is important to elucidate the intracellular fate and potential functions of IL-1β in this cell type.

To conclude, the mechanism that mediate IL-1β activation in HASMCs after IL-1R1 ligation are atypical and require only 1 signal. The process occurs independently of NLRP3 formation or caspase-1 activity and is driven by a serine protease, likely cathepsin G. However, we still do not understand why cleaved IL-1β is not secreted by HASMCs. This and other important aspects regarding release of IL-1β in VSMCs will be explored in the following chapter.

Summary:

- Synthesis and cleavage of IL-1β is dependent on NF-κβ activation in HASMCs treated with IL-1α.
- Cleavage of IL-1β is caspase-independent in HASMCs treated with IL-1α.
- Activation of IL-1β is carried out by serine proteases, presumably cathepsin G, in HASMCs treated with IL-1α.

5. IL-1β remains inside activated Vascular Smooth Muscle Cells

I

Introduction

Protein secretion is an essential biological process that enables cells to communicate with the surrounding environment[207]. The mechanisms utilised by cells to secrete proteins are diverse, but can be broadly classified as conventional and unconventional[208].

The conventional or classic pathway of protein secretion initiates by the binding of the signal peptide (leader sequence) of a nascent protein to the signal recognition particle which subsequently guides the protein to the endoplasmic reticulum[208, 209]. Once the protein enters in the lumen of the endoplasmic reticulum, it is packed in COPII-coated vesicles and transferred to the Golgi apparatus[208]. Finally, the protein exits the Golgi apparatus and is translocated across the plasma membrane to the extracellular compartment[209].

On the other side, leaderless and some transmembrane proteins utilise other non-classic routes to move out of cells[210]. Rabouille and collaborators classified the mechanism of unconventional secretion of proteins in 4 groups: type I, mediated by formation of pores in the plasma membrane; type II, through ABC transporters, type III, dependent on autophagosome and endosome formation; and type IV, direct form the ER, bypassing the Golgi apparatus[208, 210]. Multiple proteins are secreted using this unconventional pathway including: FGF2, Atg proteins, migration inhibitory factor, hydrophilic acylated surface proteins and IL-1β[208, 211].

Concerning IL-1β, both the proform and cleaved forms lack a signal peptide. Previous studies have demonstrated that immune cells secrete mature IL-1β through different mechanisms such as exocytosis via exosomes and lysosomes, shedding of microvesicles after autophagy induction and via membrane pore formation (see chapter 1).

In VSMCs, initial investigations affirmed that caspase-1 does not cleave pro-IL-1β as a consequence of the inhibitory effect of serpin B9, and therefore pro-IL-1β remains inside cells[118, 119]. Later, Schönbeck and collaborators reported that VSMCs treated with CD40 ligand are able to secrete mature IL-1β into the extracellular compartment, based on the results of thymocyte bioassays. Nevertheless, IL-1β presence in the conditioned media was not confirmed by radioimmunoprecipitation or Western blot analysis[120].

Likewise, other studies demonstrated that IL-1β is secreted by VSMCs after treatment with oxidized low density lipoproteins, β-glycerophosphate, high mobility group box 1, or Leu-Leu-O-methyl ester[121-124], but the process that mediate the release of IL-1β in these conditions was not described. Recently, the group led by Proudfoot also determined that VSMCs release IL-1β through exosomes after incubation with calcium phosphate particles[125].

Regarding these investigations, it is important to highlight some aspects. In the study that used β-glycerophosphate to induce calcification in VSMCs[121], IL-1β exit was triggered after 2 days in culture, and cell viability was not determined. Thus, I cannot conclude if the concentrations of IL-1β found in the CM are caused by secretion or rupture of the VSMC plasma membrane.

In addition, the group led by Kim affirmed that incubation with high mobility group box 1 causes secretion of IL-1β by VSMCs[122]. However, the highest IL-1β concentration detected in the CM after treatment was a negligible 15 pg/mL, which is close to the limit of detection of the ELISA.

Finally, the level of IL-1β found in the CM after inducing lysosomal membrane permeabilization was more significant in some experiments[123], but in these cases the reduction in cell viability was higher than 50%, which probably indicates that the IL-1β in the conditioned media was a consequence of cellular lysis.

In contrast, our data described above demonstrates that IL-1β is not secreted by HASMCs after incubation with IL-1R1 or TLR4 ligands (chapter 3, 3.2), or common inflammasome activators such as ATP, MSU crystals or nigericin (chapter 3, 3.8). I also excluded that serpin B9 was the reason for cleaved IL-1β remaining in the cytosol of VSMCs (chapter 4, 4.3).

Therefore, this chapter reports the experiments carried out to investigate other hypotheses that could explain why there is no release of IL-1β from HASMCs.

Aims:

1. To investigate if HASMCs contain the pore-forming protein gasdermin D.

2. To analyse the effect of LPS transfection on IL-1β release from HASMCs.

3. To study the biological activity of IL-1β from activated HASMCs.

4. To determine if HASMC-derived IL-1β is complexed to an inhibitor or binding partner.

5.1 Failure of activated HASMCs to release mature IL-1β is not due to an absence of Gasdermin D.

Gasdermin D is a protein that after cleavage by caspase-1, -4, -5, or -11 binds to phosphatidylinositol and phosphatidylserine within the cellular membrane and leads to pore formation and release of IL-1β from immune cells[84, 85]. Thus, I wondered whether a lack of gasdermin D in HASMCs was the reason they do not secrete IL-1β. I analysed gene expression in HASMCs through qPCR, which confirmed that they express *GSDMD* due to a low Ct value of ~21 (Fig. 36A), and that treatment with IL-1α does not appear to modify *GSDMD* expression. Moreover, I studied the presence of this protein inside HASMCs using Western blotting, but to carry out this experiment I first assessed the capability and concentration of the antibody required to detect gasdermin D in Thp1 cells (Fig. 36B,C). I observed that full-length gasdermin D (53 kDa) is present in Thp1 cells, and that the ideal concentration of the primary antibody for detection is 0.36µg/mL (Fig. 36C). I investigated expression of gasdermin D protein in HASMCs, which showed that HASMCs possess the full-length gasdermin D as well (Fig. 36D).

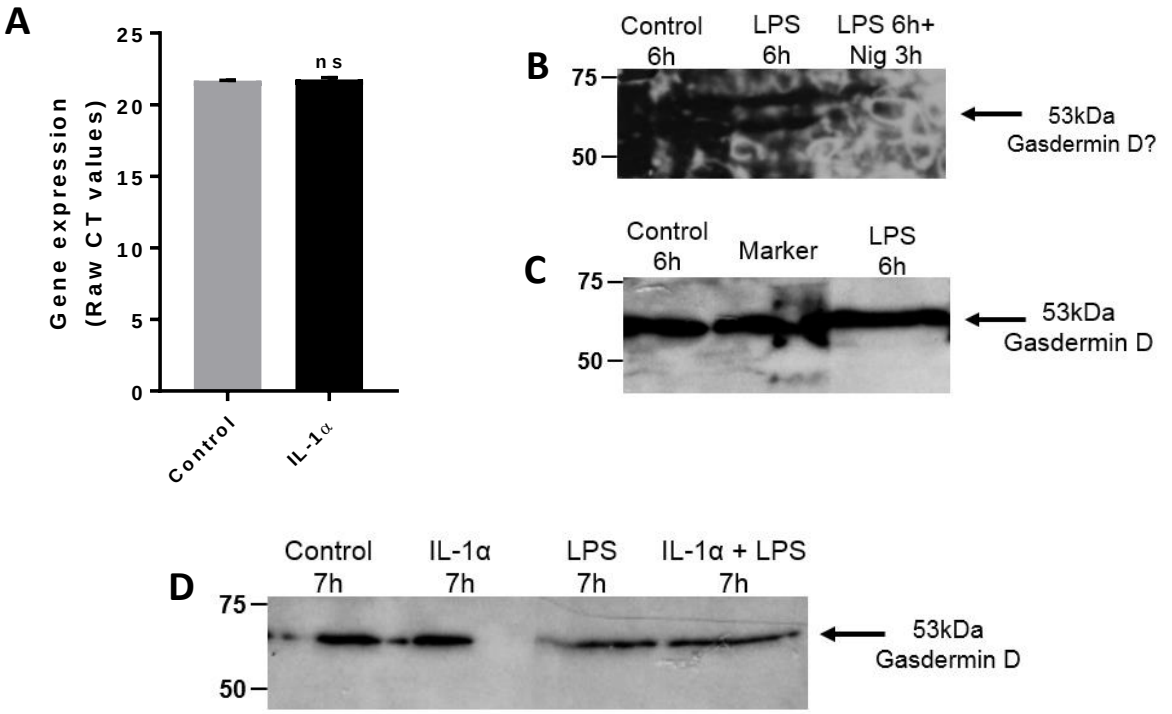


Fig. 36. HASMCs express Gasdermin D. (A) qPCR data showing expression of *GSDMD* transcript by HASMCs treated ±IL-1α (7 h), as indicated. **(B,C)** Western blots for Gasdermin D in Thp1 lysates after treatment with LPS ± Nigericin for the time indicated, and a concentration of the primary antibody of 1.08µg/mL **(B)** or 0.36µg/mL **(C)**. **(D)** Western blot for Gasdermin D in HASMC lysates after treatment with ±IL-1α and/or ±LPS for the time indicated, using a concentration of the primary antibody of 0.36µg/mL. Data represents mean ± SEM, n = 2 (A), 2 (D); p = ns > 0.05.

5.2 Overexpression of NT-Gasdermin D does not cause release of IL-1β into the CM.

After confirming gene and protein expression of gasdermin D in HASMCs, I studied whether it could cause HASMC death and secretion of IL-1β, or whether an inhibitor could block gasdermin D function. I did this by overexpressing the active N-terminal portion of the protein. Initially, I expressed NT-*GSDMD* in HeLa cells and observed that the cells died (Fig. 37), proving that the vector worked as planned. Next, I overexpressed NT-*GSDMD* in HASMCs and confirmed through microscopic imaging and PI staining that the majority of cells underwent cell death (Fig. 38). Additionally, HASMCs were stimulated with IL-1α/LPS after NT-Gasdermin D transfection (Fig. 39A) and the concentrations of IL-1β in the CM ±NP-40 was determined by ELISA (Fig. 39B). The results showed absence of IL-1β release (free or inside vesicles) in all samples analysed. Therefore, cell death can be induced in HASMCs after NT-Gasdermin D transfection, but it does not lead to secretion of IL-1β.

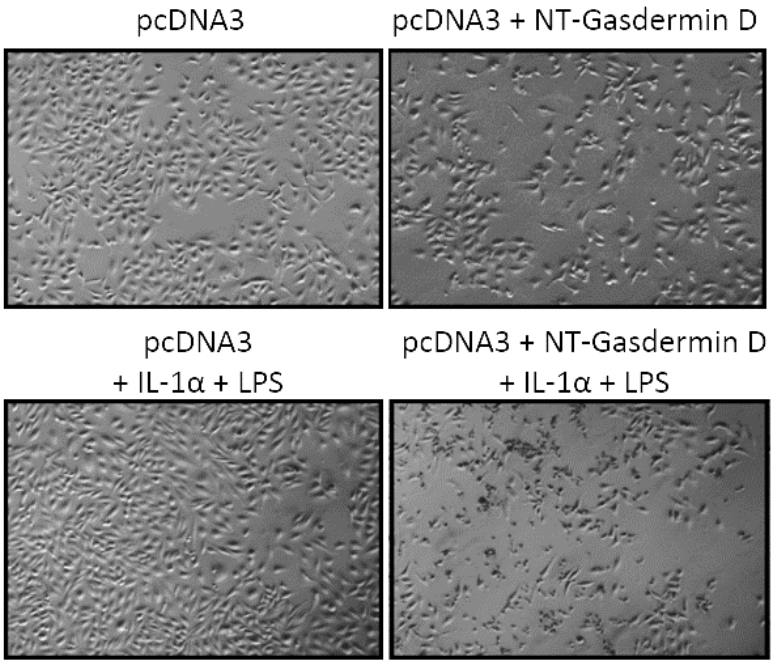


Fig. 37. Transfection of an NT-Gasdermin D expression vector induces cell death in HeLa cells. Microscopic images of HeLa cells after transfection of pcDNA3 ± NT-Gasdermin D (24 h) and treatment ± IL-1/LPS (6 h), as indicated.

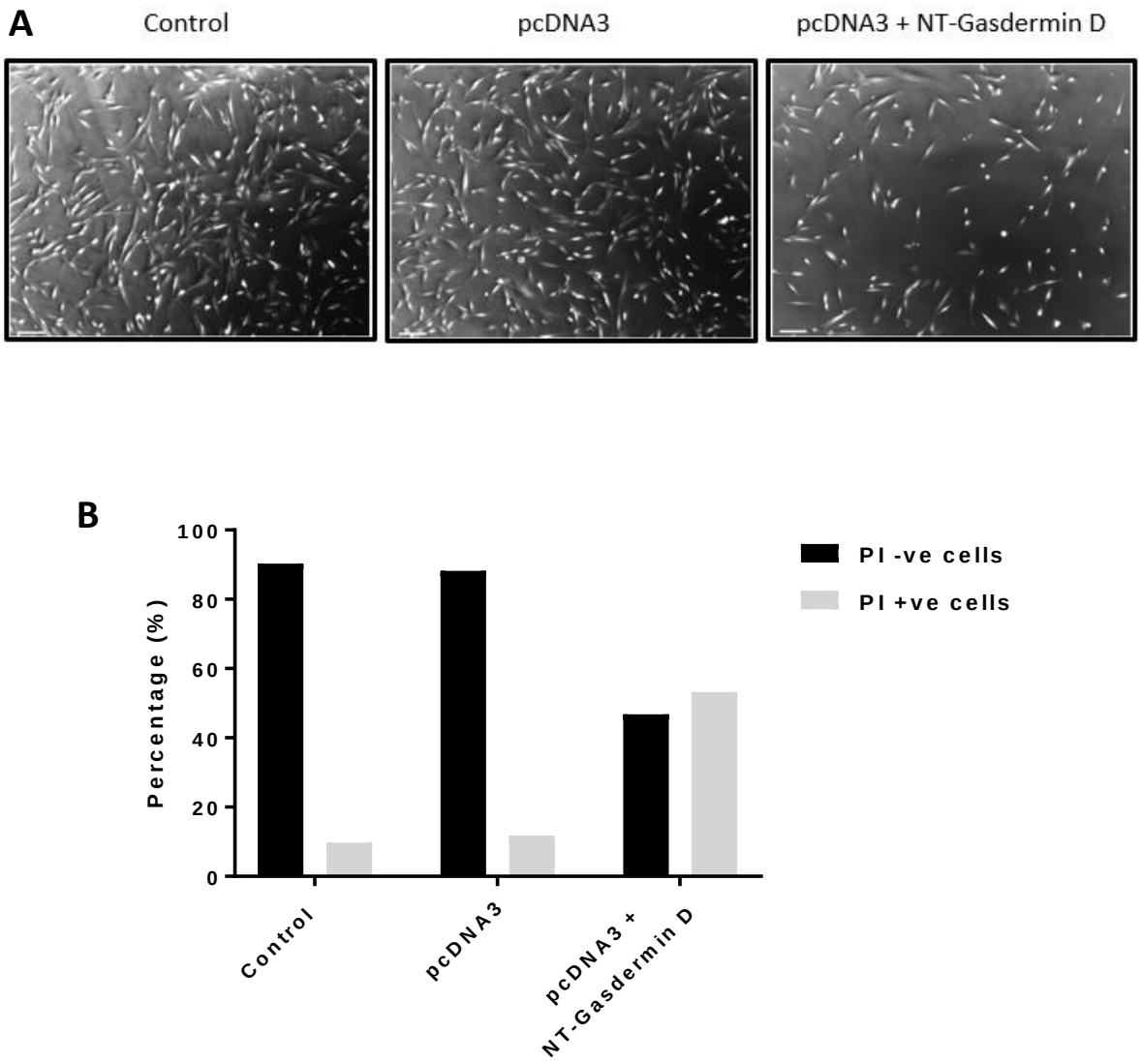


Fig. 38. Transfection of NT-Gasdermin D induces cell death in HASMCs. (A) Microscopic images of HASMCs after transfection of pcDNA3 ± NT-Gasdermin D (24 h), as indicated. **(B)** Percentage of PI -ve and PI +ve cells after transfection of pcDNA3 ± NT-Gasdermin D (24 h), as indicated.

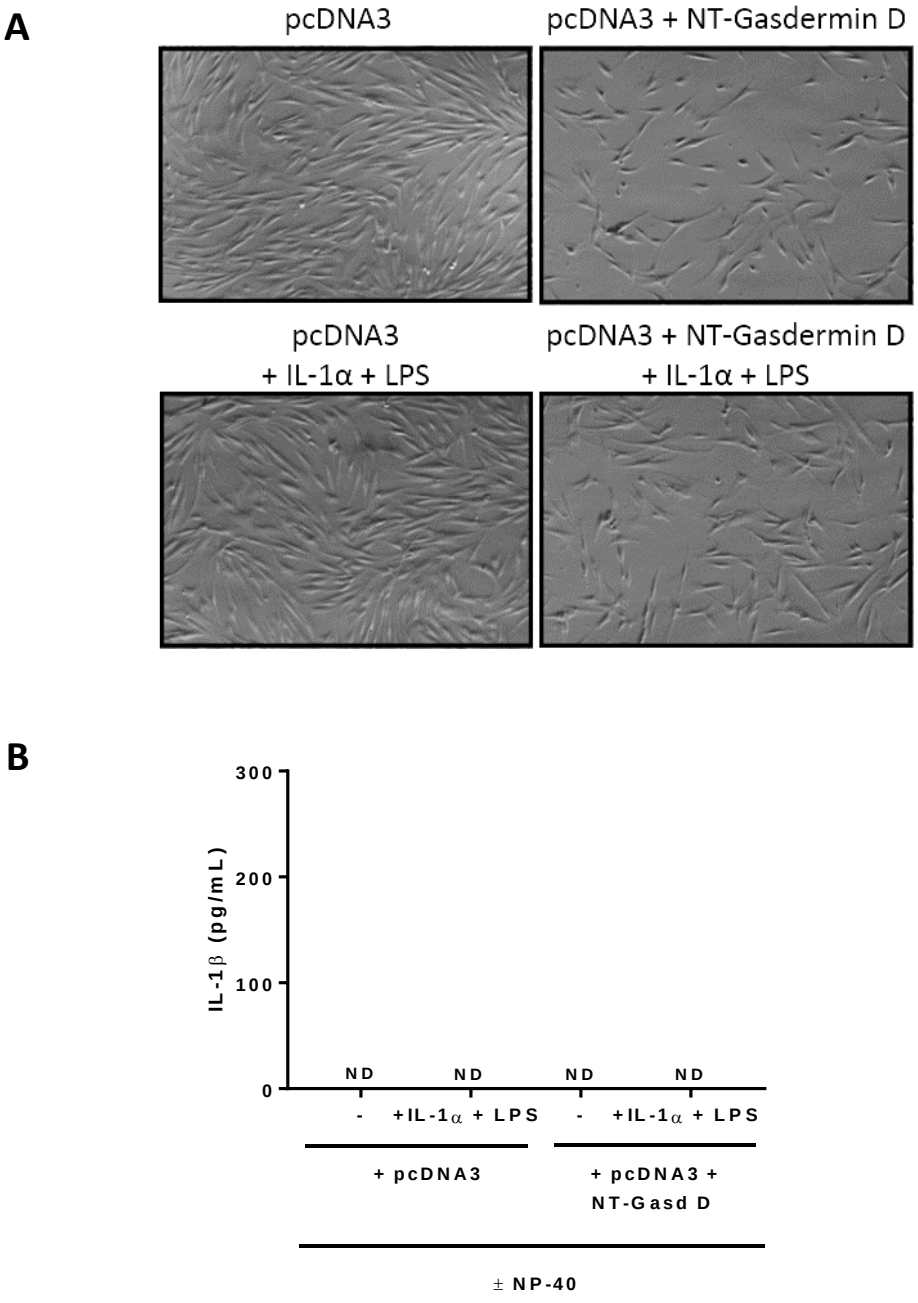


Fig. 39. IL-1β is not found in the CM of HASMCs after induction of cell death using NT-Gasdermin D. **(A)** Microscopic images of HASMCs after transfection of pcDNA3 ± NT-Gasdermin D (24 h) and treated ± IL-1/LPS (6 h), as indicated. **(B)** ELISA data showing level of mature IL-1β in the CM of HASMCs ± NP-40 after transfection of pcDNA3 ± NT-Gasdermin D (24 h) and treated ± IL-1/LPS (6 h), as indicated. ND = not detected. n = 5.

5.3 IL-1β is detected in the CM of HASMCs after detergent permeabilization.

IL-1β is not detected in the CM after expression of NT gasdermin D. Thus, I wondered if IL-1β is released from HASMCs after gasdermin D overexpression but adheres to the cell culture plates avoiding its later detection. Therefore, the presence of IL-1β was tested in the CM of IL-1α-stimulated HASMCs after cell permeabilization using different detergents (Fig. 40). I utilised NP-40, a non-ionic detergent that causes plasma membrane disruption without compromising the structure and stability of proteins[212]; saponin, a glycoside detergent that causes the formation of 8nm pores in the cell membrane, which are smaller than 10-14nm NT-Gasdermin D pores (IL-1β is approximately 4.5nm diameter)[47, 213-215]; and finally, Biolegend Intracellular Staining Permeabilization Wash Buffer, which is typically used in the process of intracellular cytokine staining. The results confirmed that mature IL-1β can be detected in the CM of HASMCs by ELISA when the cell membrane is deliberately permeabilised with detergents, excluding the presence of artefacts in the previous analysis.

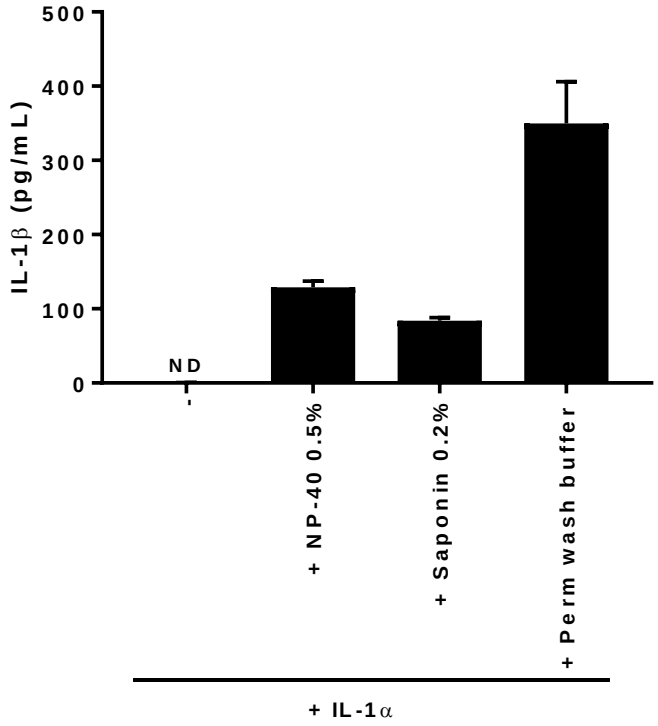


Fig. 40. IL-1β is present in the CM of IL-1α-treated HASMCs after permeabilization of the cell membrane. ELISA data showing the level of mature IL-1β in the CM of HASMCs treated with IL-1α (7 h), followed by detergents as indicated. ND = not detected. Data represents mean ± SEM. n = 2.

5.4 Experimental VSMC phenotypic switching does not lead to IL-1β release

VSMCs are characterised by possessing high plasticity and different stimuli can modulate the phenotype of VSMCs from a contractile or differentiated state to a synthetic or dedifferentiated state, which has been associated with atherosclerosis and vascular restenosis[128]. The phenotypic switch can modify the morphology of cells, as well as altering several cellular processes including division, migration and synthesis of proteins[216]. Therefore, I studied the production and release of IL-1β from HASMCs treated with IL-1α/LPS after inducing phenotypic change. HASMCs were predominantly in a contractile state before treatment (based on the morphological characteristics of cells in culture), then PDGF-β or IFNγ were used to promote a synthetic phenotype[217, 218], TGF-β to induce a contractile phenotype[219], cholesterol to stimulate foam cell transformation[220] and BMP-2/9 to modulate osteoblastic transdifferentiation[221, 222].

The results showed that IL-1β is not detected in the CM of HASMCs upon stimulation with any of the treatments previously mentioned (Fig. 41A-F). Likewise, mature IL-1β generation inside HASMCs does not increase after PDGF-β, TGF-β and IFNγ pretreatment (Fig. 42A-C). However, I observed a decrease in the levels of IL-1β produced by HASMCs pretreated with cholesterol (Fig. 42D) and an increment of IL-1β concentration after BMP-2/-9 induction (Fig. 42E,F). However, these differences were likely explained by either a reduction in cell density with Chol (Fig. 43A) or an increase cell density with BMP-2 and BMP-9 (Fig. 43B,C). Thus, treatment with the cytokines reported does not result in HASMCs that can secrete mature IL-1β after activation.

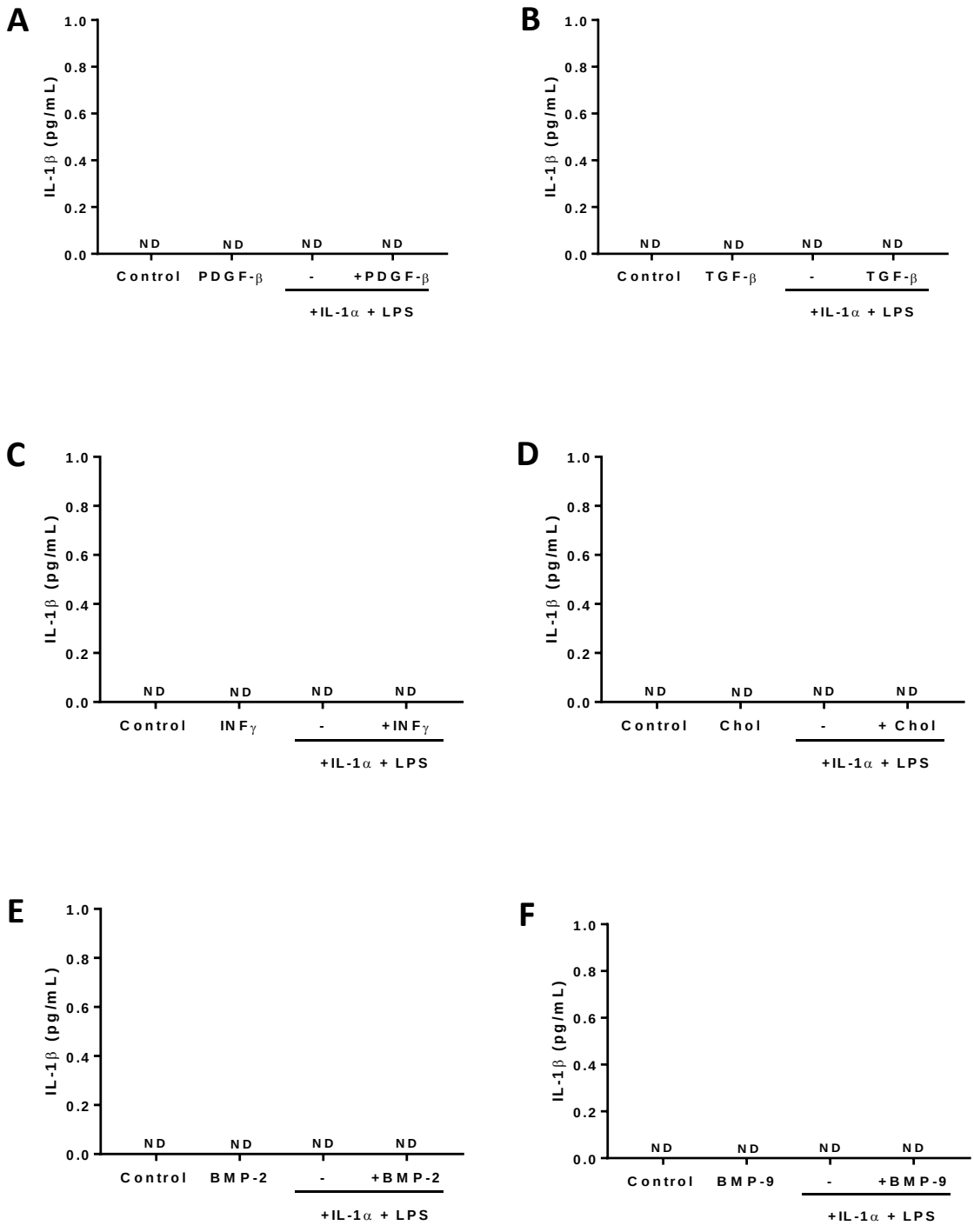


Fig. 41. IL-1β is not released into the CM of HASMCs after treatment with diverse cytokines. (A-F) ELISA data showing the level of mature IL-1β in the CM of HASMCs stimulated with ± IL-1α/LPS (6 h), after pretreatment with PDGF-β (3 days) **(A)**, TGF-β (3 days) **(B)**, IFNγ (18 h) **(C)**, cholesterol (3 days) **(D)**, BMP-2 (2 days) **(E)**, BMP-9 (2 days) **(F)**, as indicated. ND = not detected. n = 3 (A-F).

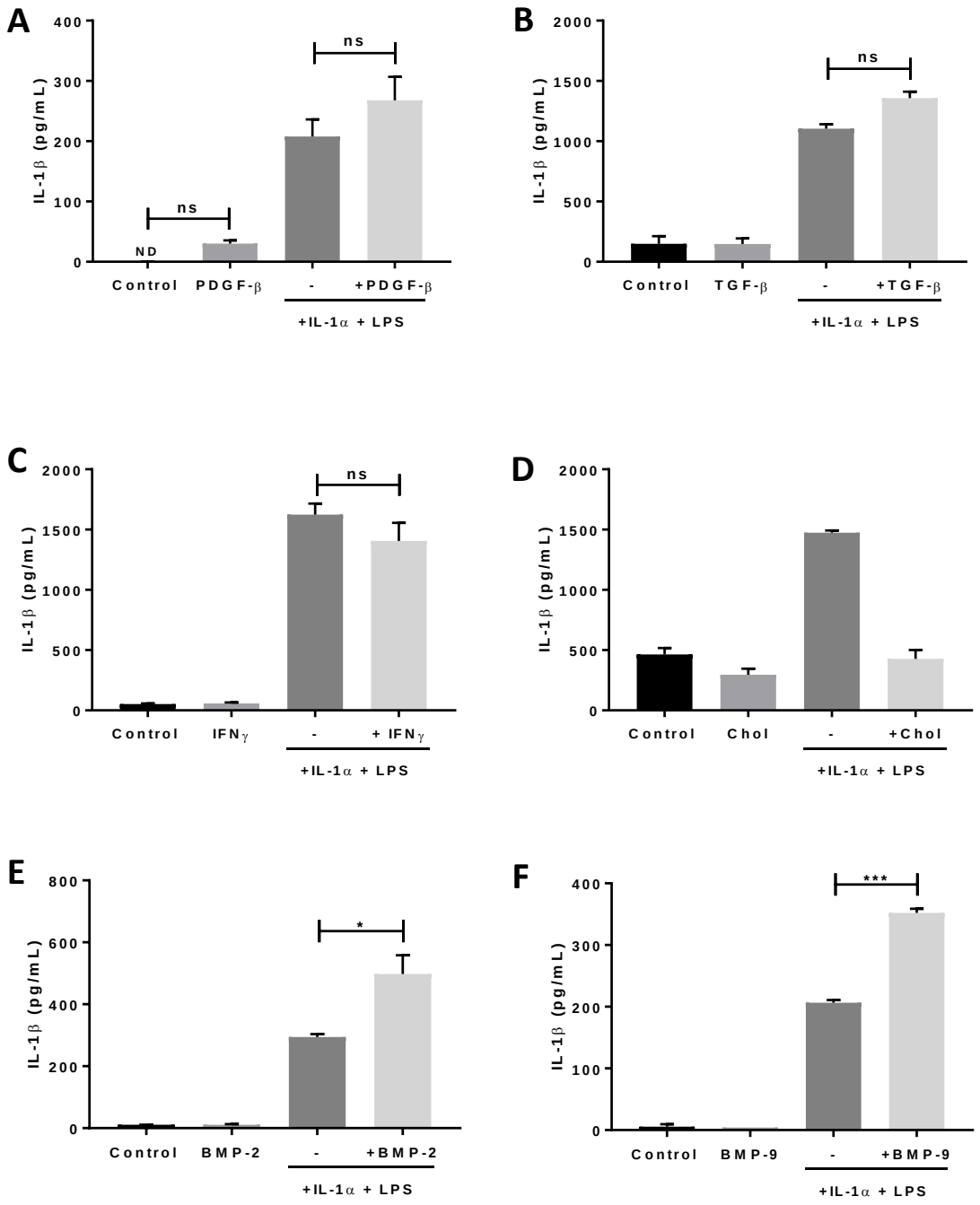


Fig. 42. Mature IL-1β generation is not released into the CM of HASMCs after treatment with diverse cytokines. (A-F) ELISA data showing the level of mature IL-1β inside HASMCs stimulated with ± IL-1α/LPS (6 h), after pretreatment with PDGF-β (3 days) **(A)**, TGF-β (3 days) **(B)**, IFNγ (18 h) **(C)**, cholesterol (3 days) **(D)**, BMP-2 (2 days) **(E)**, BMP-9 (2 days) **(F)**, as indicated. ND = not detected. Data represents mean ± SEM, n = 3 (A-F); p = ns > 0.05, * ≤0.05, ***≤0.001.

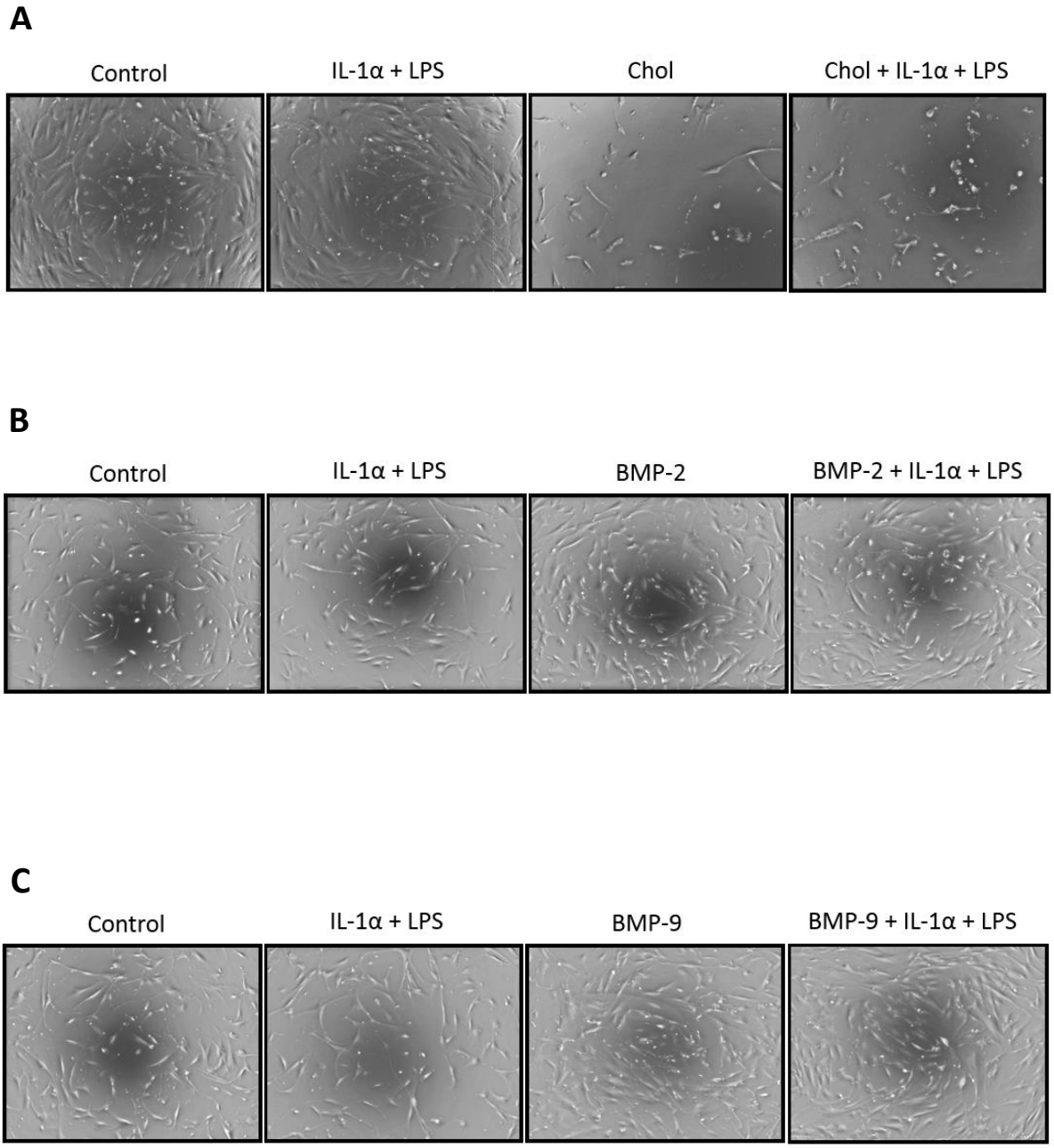


Fig. 43. Cholesterol and BMP-2/9 treatment affects HASMC density. (A-C) Microscopic images of HASMCs after treatment with cholesterol (3 days) **(A)**, BMP-2 (2 days) **(B)** or BMP-9 (2 days) **(C)**.

5.5 Low concentrations of IL-1β are detected in the CM of HASMCs treated with proapoptotic stimuli.

Apoptosis of VSMCs is associated with development of aneurysms[223, 224], restenosis after angioplasty[225], and atherosclerosis, where it promotes plaque instability, coagulation, medial degeneration and calcification[41, 226]. I studied if HASMCs pretreated with IL-1α/LPS release IL-1β into the CM after incubation with the proapoptotic agents cycloheximide/Fas-ligand. The results show negligible concentrations of IL-1β in the conditioned media of HASMCs treated with the vehicles (glycerine and ethanol) or cycloheximide/Fas-ligand after IL-1α+LPS incubation, and there were not significant differences between the groups (Fig. 44). This suggests that treatment with proapoptotic stimuli does not lead to IL-1β release from HASMCs, but rather the ethanol vehicle reduced integrity of the plasma membrane[227], causing release of IL-1β into the extracellular milieu.

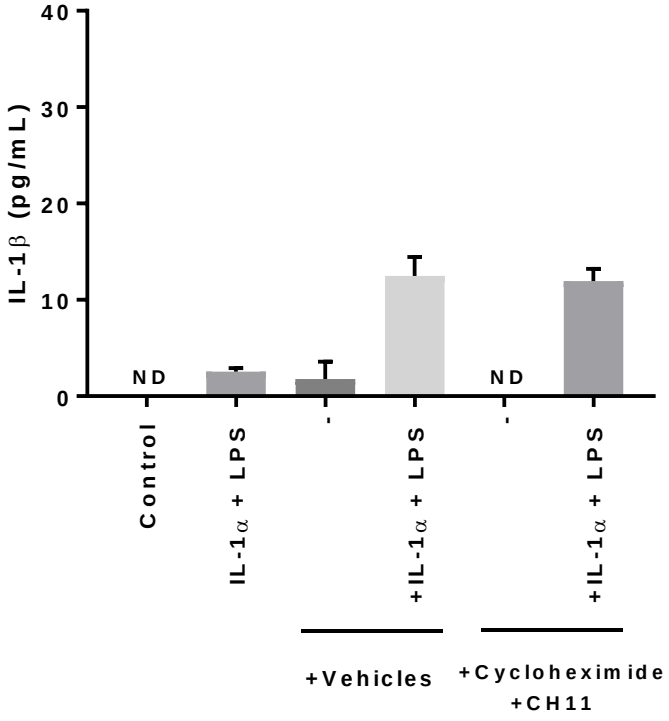


Fig. 44. IL-1β is not release into the CM of HASMCs after stimulation with proapoptotic agents. ELISA data showing the level of mature IL-1β in the CM of HASMCs ± IL-1α/LPS (7 h) and ± cycloheximide/CH11 (17 h), as indicated. ND = not detected. Data represents mean ± SEM, n = 2.

5.6 HASMCs treated with metformin do not release IL-1β.

The effect of autophagy on IL-1β is controversial. Whilst some studies found that autophagy inhibits IL-1β release, other investigations reported that this process increases its release (see chapter 1). Therefore, I stimulated HASMCs with IL-1α/LPS after preincubation with metformin, a drug used to treat type-2 diabetes mellitus that promotes autophagy through AMPK-activation and subsequent mTOR inhibition[228]. The results showed absence of IL-1β in the CM of HASMCs (Fig 45A) and no variation in the intracellular IL-1β concentration after IL-1α/LPS treatment (Fig 45B). Thus, I conclude metformin does not either increase production or lead to release of mature IL-1β from HASMCs.

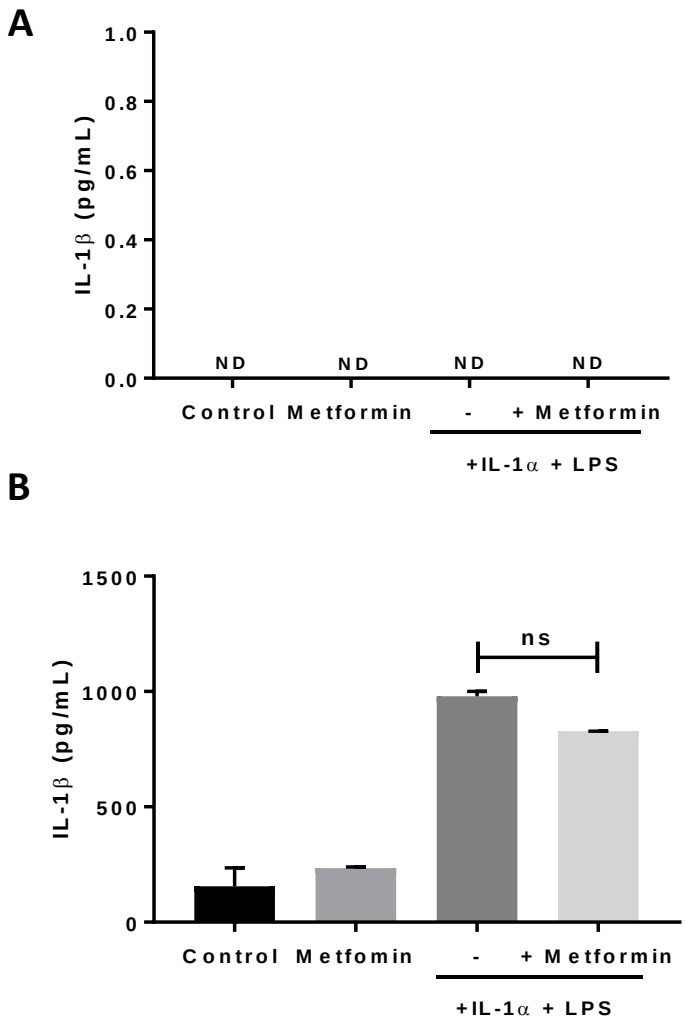


Fig. 45. IL-1β is not detected in the CM of HASMCs treated with the AMPK activator metformin. (A,B) ELISA data showing the level of mature IL-1β in the CM **(A)** and cytosol **(B)** of HASMCs treated with ± metformin (3 days) ± IL-1α/LPS (6 h), as indicated. ND = not detected. Data represents mean ± SEM, n = 3 (A,B); p = ns > 0.05.

5.7 IL-1β is not secreted after LPS transfection.

In immune cells, cytosolic LPS drives caspase-4 or -5 auto-activation (caspase-11 in mice), cleavage of Gasdermin D, followed by pyroptosis and subsequent IL-1β secretion (non-canonical inflammasome activation) - see Chapter 1. To determine if IL-1β release from HASMCs occurs after activation of the non-canonical inflammasome, I transfected LPS into untreated and IL-1α/LPS-treated HASMCs and measured IL-1β levels in the CM immediately and 6 hours after electroporation. I observed that IL-1β is not detected in the CM of untreated HASMCs (control and LPS-transfected group) (Fig. 46A). However, IL-1β was found in the CM of IL-1α/LPS-treated HASMCs immediately after transfection and slightly decreased 6 hours after, with no difference found between control and LPS-transfected group (Fig. 46B). This data suggests that IL-1β is detected in the CM of stimulated HASMCs as a consequence of the plasma membrane damage during the process of electroporation, which allows the escape of mature IL-1β that is already synthesised and contained in the cells. Thus, transfection of LPS, which normally activates the non-canonical inflammasome pathway, does not lead to IL-1β secretion by HASMCs.

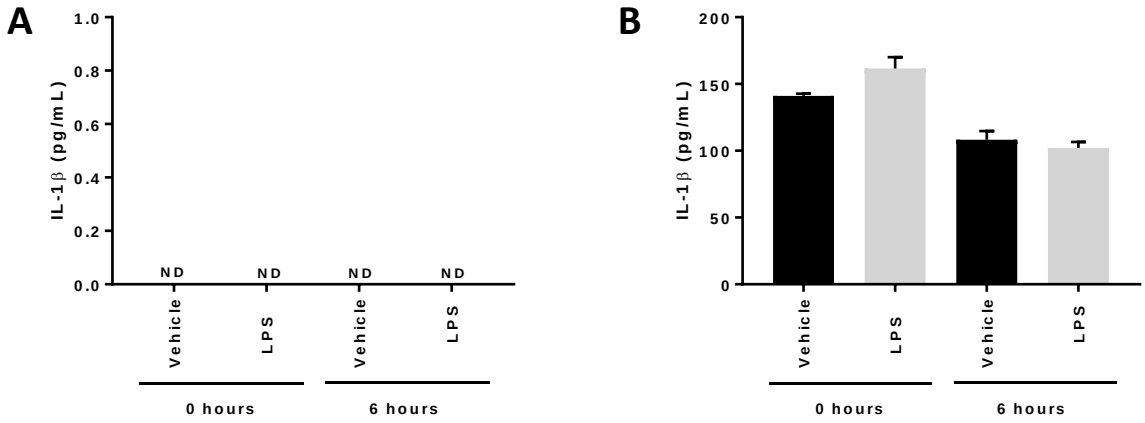


Fig. 46. Transfection of LPS does not lead to HASMC IL-1β release. (A,B) ELISA data showing the level of mature IL-1β in the CM of unstimulated **(A)** and IL-1α/LPS- stimulated **(B)** HASMCs after LPS transfection, as indicated. ND = not detected. Data represents mean ± SEM, n = 2 (A,B).

5.8 Equal quantities of mature IL-1β from HASMCs has lower cytokine activity than mature IL-1β from Thp1 cells.

IL-1 mediates dose-dependent IL-6 synthesis and secretion by HeLa cells[229], which enables HeLa cells to be used as a simple bioassay for IL-1. I used HeLa cells to establish whether the activity of the mature IL-1β inside activated HASMCs is equivalent to the mature IL-1β from Thp1 cells. To study this, I treated HASMCs with IL-1α/LPS and Thp1 cells with LPS/Nigericin, and subsequently lysed the HASMCs and Thp-1 cells using liquid nitrogen. Because IL-1β-containing HASMC lysates contained intracellular proteins, I made equivalent Thp1 lysate that consisted of cells and the IL-1β-containing CM. The level of IL-1β in these lysates was analysed by ELISA, and the lysate with the highest concentration was diluted to obtain the same amount of IL-1β in both samples. Finally, HeLa cells were treated with the lysates along with neutralising IL-1α and/or IL-1β antibodies to enable identification of IL-1β-specific activity. In the first experiments, I could not analyse the CM from the HeLa cells treated with HASMC lysates because the level of IL-6 produced exceeded the range of detection by the ELISA kit (Fig. 47A). However, neutralisation of IL-1α and IL-1β was successful in HeLa cells treated with Thp1 lysates (Fig. 47B).

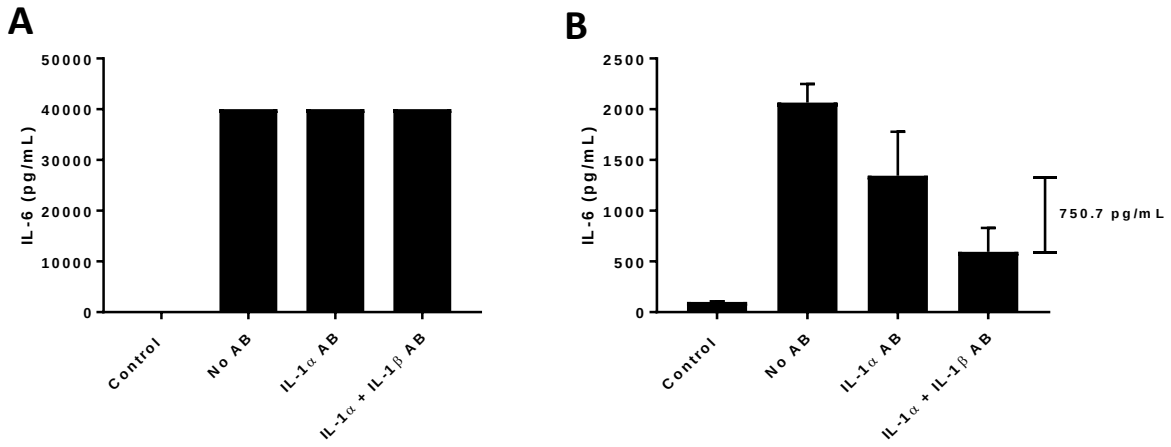


Fig. 47. Mature IL-1β from HASMCs has cytokine activity. (A,B) ELISA data showing the level of IL-6 in the CM of HeLa cells treated with HASMC lysate **(A)** or Thp1 lysate **(B)**, ± neutralising antibodies (6 h) as indicated. Data represent mean ± SEM.

As VSMCs are known to activate IL-1α upon lysis [230], I wondered whether these results were a consequence of very high levels of IL-1α in the HASMC lysate that could not be inhibited by the IL-1α neutralising antibody. Therefore, I analysed the concentration of IL-1α in the HASMC and Thp1 lysates by ELISA and found 37949 pg/ml of IL-1α in VSMC and 156pg/ml in Thp1 lysates. Hence, I titrated different concentrations of the IL-1α neutralising antibody (6, 8, 10 µg/mL) to find a concentration that could neutralise the active IL-1α and prevent the saturated

IL-6 production by the HeLa cells during the bioassay. However, none of the concentrations of IL-1α pAb used in the experiment could block IL-1α activity and the resulting IL-6 synthesis by HeLa cells (Fig. 48).

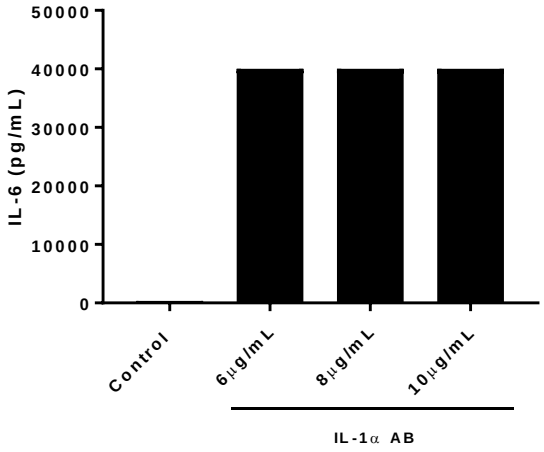


Fig. 48. No neutralizing effect on IL-1α after using different concentrations of IL-1α antibody. ELISA data showing the level of IL-6 in the CM of HeLa cells treated with HASMC lysate and 6, 8, and 10 µg/mL of neutralizing IL-1α antibody (6 h).

Therefore, during the preparation of the next HASMC lysate, I also incubated HASMCs with calpeptin, an inhibitor of calpain activity that prevents cleavage of IL-1α[231]. Later, concentrations of IL-1α were measured by ELISA and the result was lower at 19606 pg/mL. A new HeLa bioassay was performed using this HASMC lysate, but despite the blockage of IL-1α processing, the neutralization of IL-1α was still not successful (Fig. 49).

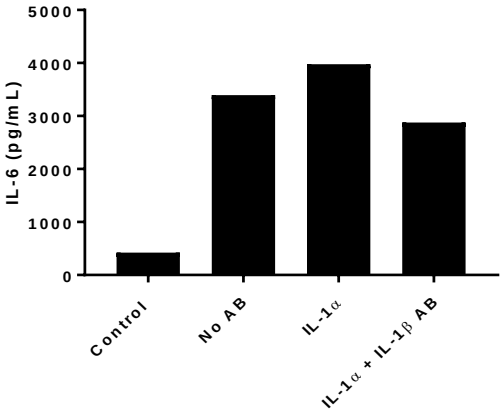


Fig. 49. Deficient neutralization of IL-1α after inhibition of IL-1α cleavage. ELISA data showing the level of IL-6 in the CM of HeLa cells treated with calpeptin HASMC lysate and 6 µg/mL of neutralizing IL-1α antibody (6h).

Thus, the next method that I followed to reduce IL-1α concentration in the HASMC lysate was immunodepletion of the sample using an IL-1α pAb and magnetic Protein G Dynabeads. After

3 rounds of immunodepletion, the concentration of IL-1α determined by ELISA was 167 pg/mL. Nevertheless, the IL-6 levels detected in the CM were still excessive (Fig. 50).

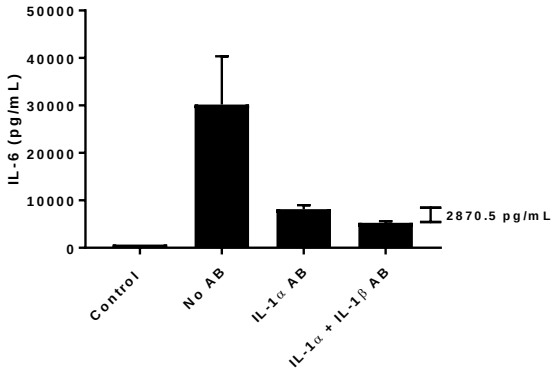


Fig. 50. Mature IL-1β from HASMCs has cytokine activity. (A,B) ELISA data showing the level of IL-6 in the CM of HeLa cells treated with HASMC lysate **(A)** or Thp1 lysate **(B)**, ± neutralising antibodies as indicated. Data represent mean ± SEM.

As VSMCs can express IL-6 in response to different stimuli[232], I realised that the IL-6 concentration found in the CM of HeLa cells might not only derived from the IL-1-induced HeLa cells, but also directly from IL-6 in the HASMC lysate. Therefore, I decided to modify the bioassay protocol by adding brefeldin A, an inhibitor of protein secretion that blocks trafficking from the reticulum endoplasmic toward the Golgi apparatus[233, 234]. Then, at the end of the bioassay the CM containing the activating IL-1 and the HASMC-derived IL-6 was washed away and the HeLa derived IL-6 released with NP-40, before testing by ELISA as before. This new experiment showed that mature IL-1β from HASMCs had ~3-fold lower cytokine activity than mature IL-1β derived from Thp1 cells (Fig. 51).

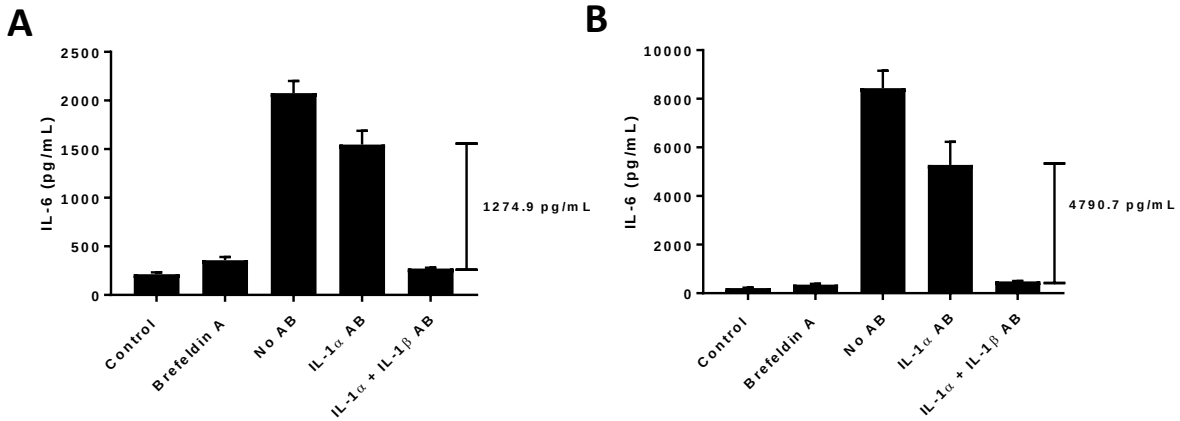


Fig. 51. Mature IL-1β from HASMCs has lower cytokine activity than mature IL-1β from Thp1. (A,B) ELISA data showing the level of IL-6 inside HeLa cells treated with HASMC lysate (A) or Thp1 lysate (B), ± neutralising antibodies (5 h) as indicated. Data represent mean ± SEM.

Furthermore, I observed a larger amount of cell debris in CM of HeLa cells treated with Thp1 lysate than HASMC lysate (Fig. 52), and wondered if this could be due to carryover of LPS/Nigericin from the Thp1 stimulation.

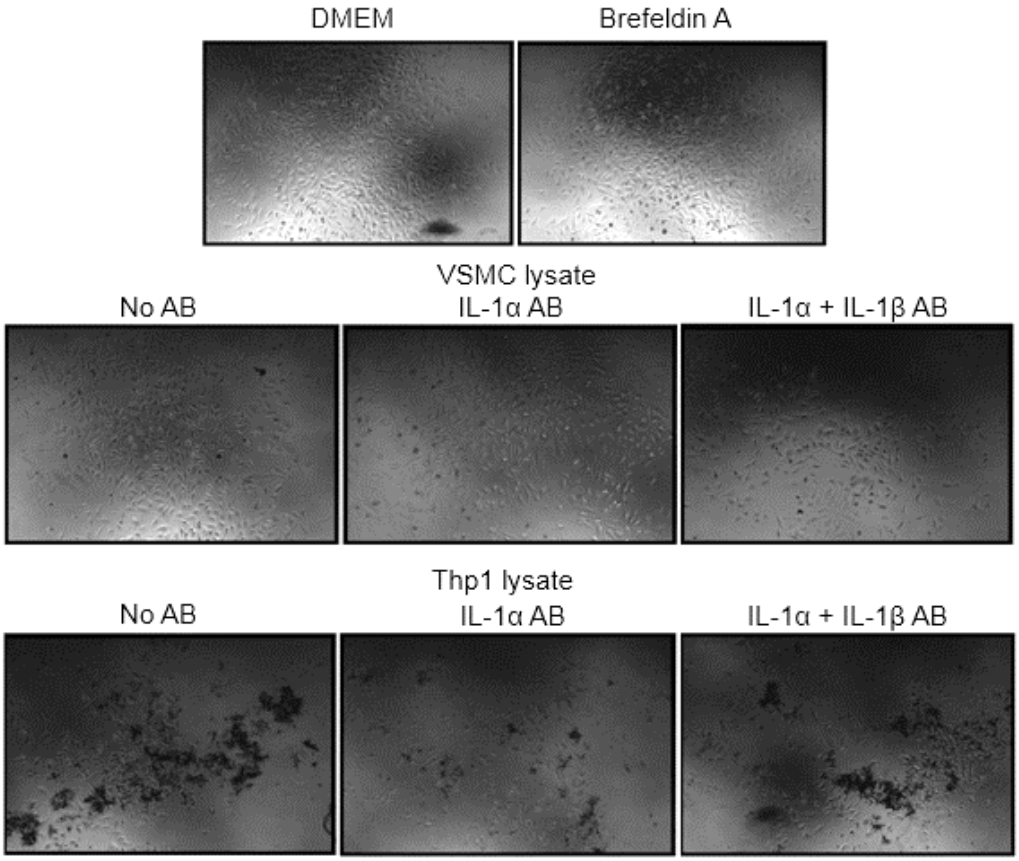


Fig. 52. Abundant cell debris in CM of HeLa cells treated with Thp1 lysate. Microscope images of HeLa cells after treatment with HASMC lysate or Thp1 lysate ± neutralising antibodies (5h) as indicated.

Therefore, I studied how much the LPS/Nigericin carryover could affect the final concentration of IL-6 produced by HeLa cells in response to direct recombinant IL-1β stimulation. The results showed only a slight reduction of IL-6 synthesis (7.1%) after treatment with LPS/Nigericin (Fig. 53).

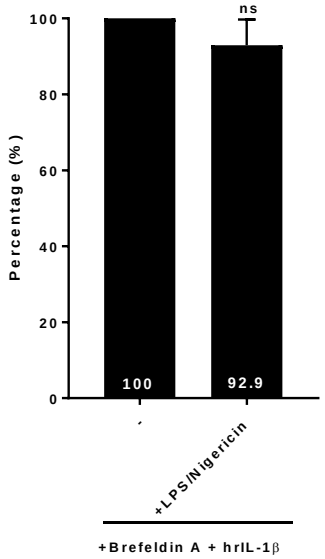


Fig. 53. Carryover of LPS/Nigericin exerts a minor effect on HeLa cell IL-6 production after IL-1β stimulation. Percentage of IL-6 production by HeLa cells with hrIL-1β ± LPS/Nigericin 'carryover' (5 h) as indicated. Data represent mean ± SEM. NS = not significant.

Additionally, I also checked if the carryover Nigericin/LPS could itself induce any IL-6 production by HeLa cells, which it did not (Fig. 54).

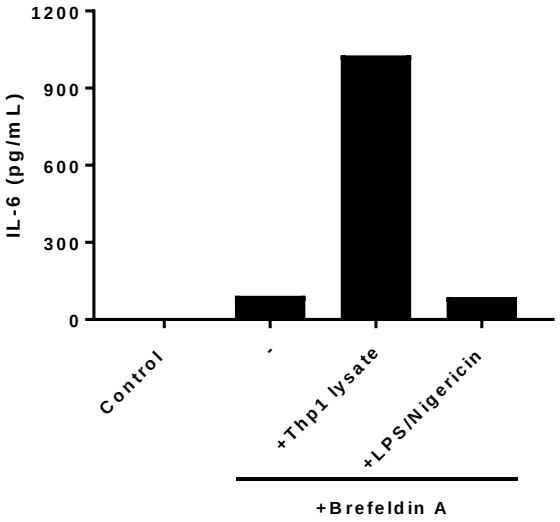


Fig. 54. LPS/Nigericin does not stimulate IL-6 production by HeLa cells. ELISA data showing the level of IL-6 inside HeLa cells treated with Thp1 lysate or LPS/Nigericin 'carryover' (5 h) as indicated.

Finally, after collating and normalising all the results from the final protocol for determining IL-1β activity, I found that the cytokine activity of mature IL-1β from HASMC lysates is 13.1-fold lower than the same amount of mature IL-1β from Thp1 cells (Fig. 55).

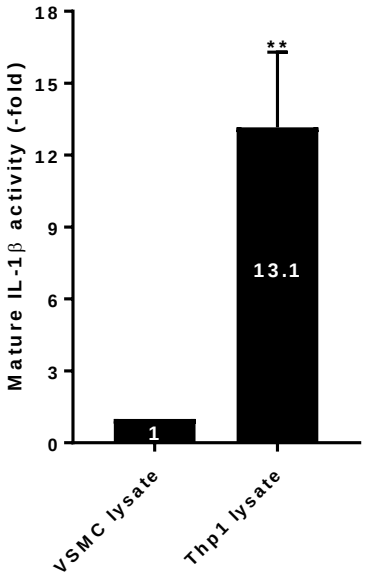


Fig. 55. Mature IL-1β from HASMCs has less activity than mature IL-1β from Thp1 cells. Relative bioactivity of equal concentrations of mature IL-1β from activated HASMCs or Thp1 cells. Data represents mean ± SEM, n = 4; p = **≤ 0.01.

5.9 Mature IL-1β inside HASMCs does not possess a stable binding partner.

Mature IL-1β is generated but not secreted by HASMCs and exhibits reduced cytokine activity compared to IL-1β from Thp1 cells, as shown above. These findings could be explained by the existence of a binding partner that establishes a strong interaction with mature IL-1β inside HASMCs. This could both block secretion and result in the lower cytokine activity witnessed. To study this hypothesis, I performed size fractionation of HASMC lysates using gel filtration. However, the majority of IL-1β eluted off the column in the fraction corresponding to the expected size of mature IL-1β (Fig. 56), instead of an earlier fraction that would indicate the presence of a binding partner that had increased the molecular size and weight of a complex. Additionally, I estimated protein recovery to exclude loss of IL-1β on the column during filtration, and a high percentage of IL-1β was detected after elution - 92% (Fig. 57). This data suggests the absence of an IL-1β binding partner, or loss of complex stability when the HASMC lysate flows through the column.

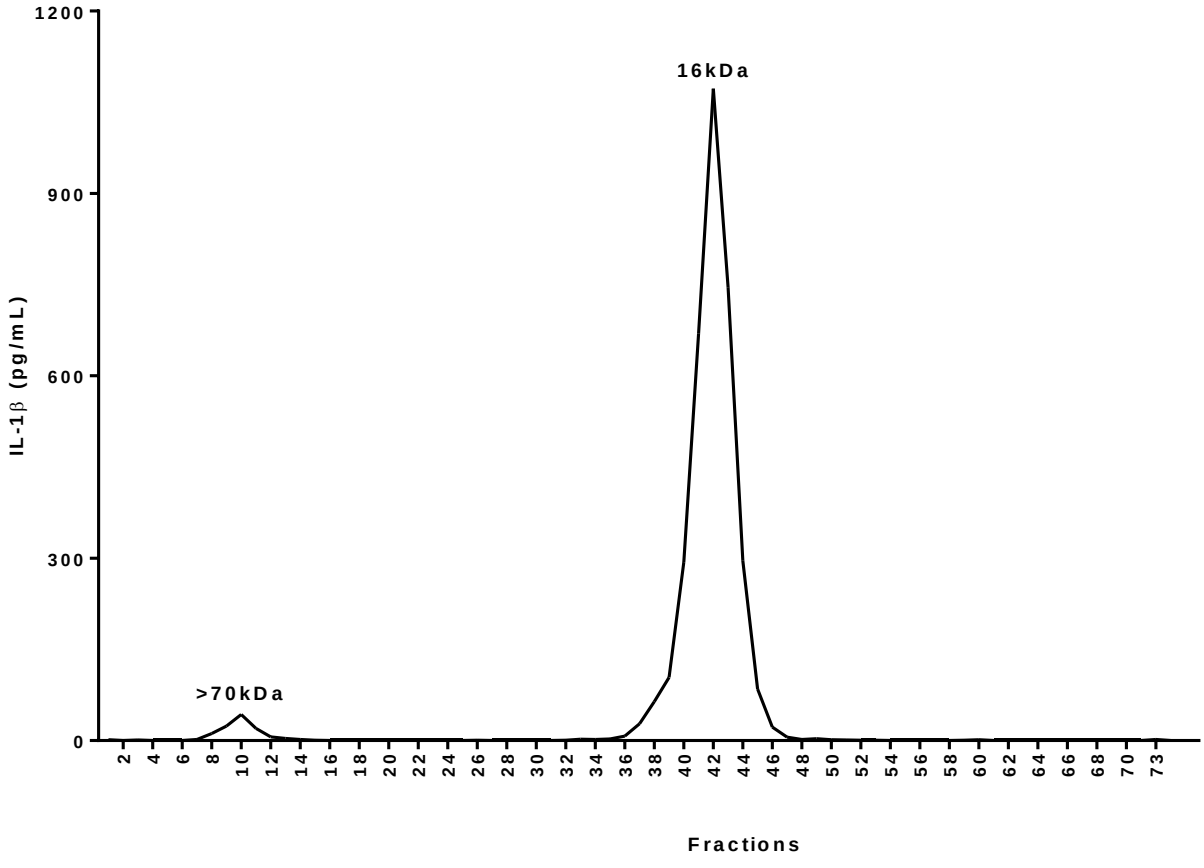


Fig. 56. IL-1β likely lacks a binding partner. ELISA data showing the level of mature IL-1β in the fractions obtained after size fractionation on a gel filtration column (Superdex 75, fraction range 3000Da to 70000Da). Representative of n = 3.

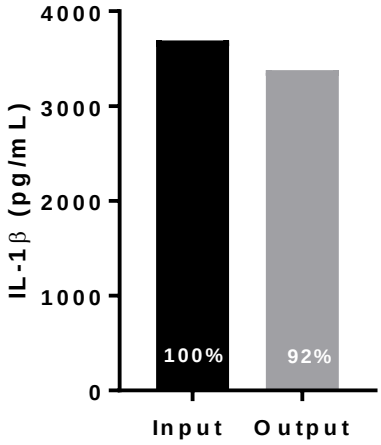


Fig. 57. The majority of input IL-1β is recovered after size fractionation. Graph showing the input and output of mature IL-1β before or after size fractionation.

5.10 IL-1β is detected in the CM of HASMCs after IL-1β overexpression.

In macrophages, transfection of mature IL-1β, but not pro-IL-1β, leads to release of p17 into the conditioned media[116]. Thus, I studied if overexpression of IL-1β in HASMCs could overcome the potential mechanism that blocks its secretion (e.g. by titrating out an inhibitor or binding partner), allowing the subsequent appearance of IL-1β in the CM. For this purpose, I transfected pro-IL-1β and mature IL-1β into HASMCs, followed by analysis of IL-1β concentrations in the CM. Surprisingly, I detected mature IL-1β in CM after transfection of either the p17 and p31 form (Fig. 58A). Likewise, our evidence supports the absence of a binding partner of IL-1β due to the fact that the relationship between the levels of mature IL-1β in the conditioned media and p17 IL-1β DNA concentration is ~linear (Fig. 58B). In contrast, presence of a binding partner would likely show no release of IL-1β until a certain threshold, whereby IL-1β concentration would exceed that of a potential inhibitor or binding partner.

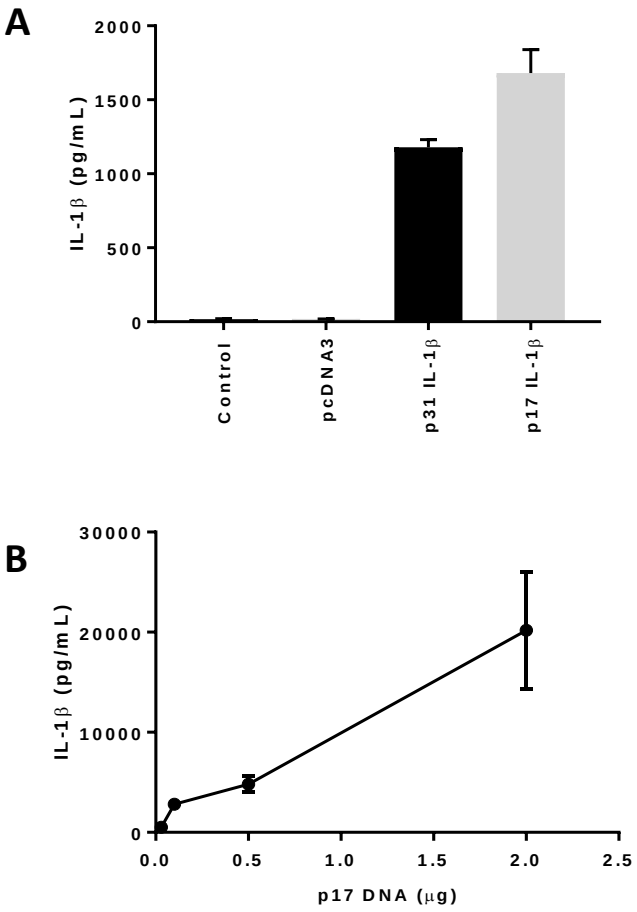


Fig. 58. IL-1β is detected in the CM of HASMCs after IL-1β overexpression. (A) ELISA data showing the level of mature IL-1β in the CM of HASMCs after transfection of pcDNA3 containing p17 or p31 IL-1β cDNA (24 h), as indicated. **(B)** ELISA data showing the level of IL-1β in the CM of HASMCs after transfection of increasing amounts of pcDNA3 containing p17 IL-1β (30 h), as indicated. ND = not detected. Data represents mean ± SEM, n = 2 (A,B).

Discussion

IL-1β is a leaderless protein that is secreted to the extracellular compartment through unconventional pathways without requirement for the endoplasmic reticulum-Golgi complex[47]. Multiple studies have characterised the machinery and the mechanisms implicated in the release of IL-1β from immune cells, including secretion through exosomes, shedding of microvesicles, after autophagy induction and pyroptosis[47]. In contrast, preceding investigations about the release of IL-1β from VSMCs are contradictory, with initial studies affirming that VSMCs do not process and release IL-1β, and following investigations reporting that IL-1β can be secreted after stimulation with diverse stimuli (e.g. oxidized low density lipoproteins, β-glycerophosphate, high mobility group box 1, Leu-Leu-O-methyl ester, calcium phosphate particles[118, 119, 121-125]). However, in some of these studies the presence of IL-1β is likely a consequence of rupture of the plasma membrane or the level of IL-1β in the conditioned media is negligible.

The results in this chapter show that induction of cell death after transfection of NT-Gasdermin D or treatment with proapoptotic stimuli do not trigger IL-1β release from HASMCs. Likewise, HASMCs pretreated with PDGF-β, TGF-β, IFNγ, cholesterol, or BMP-2/-9, to promote an altered phenotype, or treated with the AMPK activator metformin do not secrete IL-1β. Similarly, IL-1β was absent in the conditioned media of HASMCs transfected with LPS to activate the non-canonical inflammasome. Interestingly, IL-1β is detected in the conditioned media of IL-1α-stimulated HASMCs after deliberate permeabilisation with detergent. Mature IL-1β from HASMCs possess lower biological activity in comparison to IL-1β from Thp1 cells, but this is unlikely to be due to an IL-1β inhibitor or binding partner.

These findings show that failure of HASMCs to release mature IL-1β does not occur exclusively in response to IL-1R1 and/or TLR4 ligation, but is also evidenced in HASMCs treated with other stimuli (e.g. intracellular LPS) that are known to trigger release of IL-1β from macrophages. Moreover, the reduced biological activity of mature IL-1β from HASMCs compared to Thp1 cells supports our data suggesting that pro-IL-1β is likely cleaved by an atypical serine protease, given that previous investigations have demonstrated that IL-1β proteolysis by elastase, proteinase 3 and cathepsin G exhibits lower biological activity in contrast to IL-1β processed by caspase-1[235].

Concerning the reason why IL-1β is not release from HASMCs into the extracellular milieu, our results rule out secretion of IL-1β through exosomes, lysosomes or microvesicles, given that detergent treatment of conditioned media did not increase detectable IL-1β (see chapter 3,

3.5). Similarly, HASMCs treated with the autophagy inducer metformin do not release IL-1β. Therefore, I explored other potential hypotheses that could explain this phenomenon. As inhibitors of IL-1β activity are described in other tissues (e.g. uromodulin from pregnancy urine, UV-inducible epidermal-contra-IL-1 molecule and epidermal IL-1 inhibitor from psoriatic skins)[236-238], I studied if a binding partner or inhibitor of IL-1β in HASMCs both prevented release and reduced activity of VSMC-derived mature IL-1β. However, fractionation of HASMC lysates through a size exclusion column resulted in elution of mature IL-1β around the expected 17 kDa size, suggesting a binding partner is not present, or that the interaction is lost once cells are lysed.

Likewise, I investigated if HASMCs express gasdermin D. Gasdermin D cleavage by caspase-1 releases the N-terminal, which binds to the plasma membrane and forms pores that allow IL-1β to exit the cell. Interestingly, I found that HASMCs both express gasdermin D and undergo cell death when the active N-terminal is overexpressed, suggesting lack of IL-1β release from HASMCs is not due to defects in the gasdermin D pathway. However, because IL-1β processing in activated HASMCs is inflammasome- and caspase-independent, gasdermin D is presumably not processed and thus does not permeabilise cells to enable escape of mature IL-1β. It is important to mention that a recent study reported that human gasdermin D can be cleaved by purified cathepsin G[202]. However, this is controversial due to the fact a previous investigation demonstrated that recombinant cathepsin G did not cleave recombinant gasdermin D[239].

Another mechanism for IL-1β secretion independent of caspase-1 and gasdermin D has been described by Schroder and collaborators[116]. According to this study the cleavage of pro-IL-1β by caspase-1 is sufficient to cause release of IL-1β from macrophages in a slow manner[116]. Processing of IL-1β modifies the net charge from negatively charged pro-IL-1β to positively charged mature IL-1β, which is attracted to the negatively charged projections and ruffles of the plasma membrane, allowing mature IL-1β to translocate across to the extracellular compartment[116]. Thus, I hypothesised that the different amino acid sequence and/or confirmation of pro-IL-1β cleaved by serine proteases would result in a differently charged mature IL-1β that perhaps cannot interact with the plasma membrane, preventing its release. However, additional experiments to confirm the presumptive role of serine proteases in IL-1β processing, as well as to corroborate the lack of activation of caspases after HASMCs activation, are key to confirm this hypothesis.

In conclusion, HASMCs synthesise and cleave pro-IL-1β, but the resulting mature IL-1β remains in the intracellular space, potentially as a consequence of a lack of gasdermin D activation. To determine if an alteration of this phenomenon that normally prevents IL-1β

release from HASMCs occurs during the formation and/or destabilisation of atheromatous lesions is important to increase our understanding of the pathophysiology of cardiovascular diseases.

Summary:

- IL-1β is not release from activated HASMCs after overexpression of Gasdermin D, treatment with inducers of phenotypic switch or autophagy.
- IL-1β does not possess a binding partner in HASMCs.
- IL-1β derived from HASMCs has lower biological activity compared to IL-1β from Thp1 cells.
- Cell membrane permeabilization of HASMCs leads to release of IL-1β into the conditioned media.

6. General discussion

IL-1β is a powerful cytokine that belongs to the IL-1 family and mediates several processes including cellular proliferation, migration, apoptosis and inflammation[43, 240]. IL-1β has been implicated in the pathophysiology of diverse conditions including metabolic, neurodegenerative, autoimmune and cardiovascular diseases[57], and although IL-1β is mainly produced by immune cells, it is also expressed by other cell types such as VSMCs[43, 79, 80, 118-125].

The results in this dissertation show that IL-1β is synthesised and cleaved by HASMCs, but is not released. Interestingly, caspases do not mediate this cleavage, instead I currently believe cathepsin G most likely processes pro-IL-1β. However, additional experiments are required to corroborate these observations. Firstly, it would be important to investigate if *CTSG* is upregulated in HASMCs treated with IL-1α or LPS. In addition, measuring a decreased level of mature IL-1β after silencing cathepsin G, using siRNA or antisense oligonucleotides, would confirm its role in the process, supporting the observations in HASMCs treated with the serine protease inhibitor dichloroisocumarin.

Other pertinent experiments to prove this hypothesis include determining by Western blotting and ELISA if recombinant or HASMCs-derived pro-IL-1β are cleaved after incubation with recombinant cathepsin G. If the results show cleavage of pro-IL-1β, we could test the activity of the resulting fragment by measuring production of IL-6 by HeLa cells, and also compare this biological activity to that of mature IL-1β cleaved by recombinant caspase-1, or caspase-1 derived from Thp1 cells. If our hypothesis is correct, we would find that cathepsin G-cleaved IL-1β is biologically active, but much less potent than caspase-1 activated IL-1β.

Similarly, analysis of HASMCs-derived mature IL-1β by mass spectrometry would be important to establish the site where pro-IL-1β is cleaved in HASMCs. This could be accomplished by enrichment of cleaved IL-1β from HASMCs using immunoprecipitation, followed by SDS-PAGE, Coomassie staining and band excision, before mass spectrometry. Importantly, the limit of detection by mass spectrometry is 2ng - 5ng for proteins with molecular weight less than 75KDa, indicating this would be feasible from ~8 million HASMCs (~6 T150 flasks). Previous investigations indicate that cathepsin G would likely cleave pro-IL-1β at Y113[92, 196]. Subsequently, we could mutate the site of proteolysis in pro-IL-1β (e.g. Tyr to Phe) and test the ability of cathepsin G to generate mature IL-1β in cell-free systems, again using Western blotting and ELISA as a read out of cleavage. If we had mutated the correct site, I would expect failed generation of mature IL-1β.

Interestingly, our results also show that mature IL-1β remains in the intracellular space of HASMCs after stimulation with IL-1α and LPS. I hypothesise that this phenomenon occurs due

to the fact TLR4 and IL-1R1 ligation alone do not cause activation of caspases, and hence gasdermin D is not activated and the membrane pores through which IL-1β can exit the cell do not form. Intriguingly, although I triggered cell death after NT-gasdermin D transfection, IL-1β was still not released from HASMCs (Fig. 39B). However, the explanation for this result could be that the immediate induction of cell death upon expression of the NT-GSDMD interferes with the process of expression and activation of IL-1β, and thus it is not secreted to the conditioned media. Therefore, it would be interesting and much more robust to transfect HASMCs with a switchable NT-gasdermin D vector (e.g. using Tet On/Off), followed by stimulation with TLR4 or IL-1R1 ligand for 7 hours in order to allow the synthesis and cleavage of IL-1β, and finally to switch on the vector which would lead to membrane pore formation and allow the exit of IL-1β. If IL-1β normally remains inside HASMCs due to lack of gasdermin D activation, we would detect mature IL-1β in the conditioned media in this experiment. Indeed, mechanical disruption of HASMCs by freeze/thaw (Fig. 8B) or detergent lysis (Fig. 40) releases ample cleaved IL-1β.

Previous investigations in neutrophils demonstrate that the active form of cathepsin G is stored in intracytoplasmic azurophilic granules or secretory lysosomes and is released into phagosomes, or exocytosed in response to inflammatory stimuli[200, 202, 241, 242]. Although the existence of azurophilic granules in VSMCs has not been reported, VSMCs contain lysosomes. Thus, studying the localisation of IL-1β before and after cleavage, the colocalization of IL-1β and cathepsin G, and any colocalization of IL-1β and lysosomes (e.g. the membrane markers LAMP1, LAMP2 or CD63) by immunofluorescence or subcellular fractionation could help determine if IL-1β is directed to lysosomes, where it is subsequently cleaved by cathepsin G, but due to the lack of an additional specific stimulus is not secreted, but rather destroyed. These experiments could also show if IL-1β is sequestered and/or inactivated in other intracellular vesicles, such as autophagosomes, a phenomenon described previously in macrophages[114].

The purpose for retention of IL-1β in the cytosol after HASMC stimulation is unknown. However, vertebrate animals have developed intricate mechanisms and highly regulated pathways in order to neutralize and eliminate threats, while minimising excessive or chronic responses that could lead to tissue destruction[1]. Macrophages, the main source of IL-1β, are specialised cells of the immune system that express multiple receptors to recognise pathogens and tissue damage, and integrate signals from nearby tissues, phagocytosed cells, extracellular matrix components, and microvesicles[243]. If an insult is detected, macrophages secrete proinflammatory and cytotoxic mediators and can also initiate an adaptive immune response[243]. Once the pathogen is destroyed, macrophages sense signals in the tissue and secrete anti-inflammatory cytokines, lipoxins and other tissue-repair mediators that promote

the resolution of the inflammatory response[243]. Therefore, the prevention of IL-1β secretion by VSMCs could be a protective regulatory mechanism to avoid the release of high concentrations of IL-1β from non-professional immune cells that are not specialised in the recognition of invaders and lack the ability to switch from a pro-inflammatory to an anti-inflammatory state when needed. Thus, release of IL-1β form VSMCs could produce an uncontrolled, prolonged and deleterious inflammatory response. Nevertheless, if the initial insult was significant enough to compromise the integrity of the VSMCs plasma membrane, the release of IL-1β could alert the immune system or help amplify the immune response already mounted by specialised immune cells. Furthermore, another potential reason to avoid the exit of IL-1β from VSMCs is the fact that, VSMCs express IL-1R1 and therefore IL-1β release could induce expression and secretion of IL-1β and other proinflammatory molecules via autocrine signalling and lead to activation of a sustained and harmful inflammatory loop.

It is important to note that cell treatments that caused membrane permeabilization, such as detergents (Fig. 40), ethanol (Fig. 44) and electroporation (Fig. 46B), triggered release of IL-1β into the conditioned media. Thus, interaction of HASMCs with chemicals or proteins that interfere with the cell membrane integrity, even temporarily, or necrotic cell death could lead to exit of IL-1β to the CM.

Recently, Soehnlein and collaborators demonstrated that the cationic nuclear protein histone-4, which is present in neutrophil extracellular traps, preferentially interacts with the anionic plasma membrane of VSMCs (due to electrostatic forces) and induces membrane permeabilization[244]. This was observed in mouse models of atherosclerosis, and was important for driving inflammation, tissue destruction and finally destabilisation of atherosclerotic plaques. Thus, this could represent a novel mechanism for release of IL-1β from VSMCs. To investigate this, we could activate HASMCs and subsequently incubate them with histone-4 or coculture them with activated neutrophils, and determine the level of IL-1β in the conditioned media. This would show if IL-1β can exit the cell after pore formation caused by histone-4 or neutrophil extracellular traps.

Other potential stimuli that could cause HASMC permeabilization and subsequent release of IL-1β are pore-forming proteins such as streptolysin O derived from *Streptococcus pyogenes*[245], pneumolysin produced by *Streptococcus pneumoniae*[246], and perforin release by cytotoxic lymphocytes[247]. Similarly, induction of necroptosis in HASMCs using different treatments (e.g. TNFα, TRAIL) could lead to pore formation by MLKL[248] and exit of IL-1β to the extracellular milieu.

Furthermore, our results indicate that mature IL-1β from HASMCs exhibits lower biological activity compared to mature IL-1β from Thp1 cells (Fig. 55). Therefore, I performed size fractionation of activated HASMC lysates using gel filtration to investigate if IL-1β possesses a binding partner or inhibitor that prevents its secretion and reduces its biological activity (Fig. 56). The preliminary data show that IL-1β is eluted off in a fraction corresponding to the expected size of mature IL-1β, suggesting IL-1β lacks a binding partner or inhibitor. However, it would be important to study the biological activity of HASMC-derived IL-1β before and after size fractionation, because if the biological activity increased after fractionation, it could indicate the existence of a binding partner or inhibitor that losses interaction with IL-1β as the HASMC lysate flows through the column.

Likewise, I overexpressed pro-IL-1β and mature IL-1β in HASMCs and subsequently determined the concentration of mature IL-1β in the conditioned media. The results showed presence of IL-1β in the conditioned media, and I observed linearity in the relationship between levels of mature IL-1β detected and p17 IL-1β DNA concentration transfected. This suggests that IL-1β lacks a binding partner or inhibitor, because the presence of any of these elements would likely not cause secretion of IL-1β into the conditioned media until a certain DNA concentration threshold induced synthesis of enough IL-1β to surpass the binding partner or inhibitor.

Interestingly, I demonstrated that pulmonary artery SMCs do not secrete IL-1β either. Similarly, previous studies also report negligible concentrations of IL-1β in the conditioned media of human uterine SMCs incubated with TNFα, LPS, or IL-6, or under hypoxic conditions[249], or human term myometrium treated with corticotropin-releasing hormone or LPS[250]. Despite the fact that these investigations did not explore the intracellular concentrations of IL-1β, this data could indicate that IL-1β also remains in the intracellular space of these cell types. Hence, to investigate the levels of IL-1β in the conditioned media and cell lysates of activated SMCs from other localizations (e.g. gastrointestinal system, airway, uterus) is important to determine if production and failed secretion of mature IL-1β occurs in other tissues, and if the dysregulation of this mechanism could play a role in the pathogenesis of other diseases associated with inflammation.

In conclusion, HASMCs express and process IL-1β in response to TLR4 and IL-1R1 ligation, but the resulting mature IL-1β remains in the intracellular space. The reason and clinical significance of this phenomenon in physiological and pathological conditions is unknown. Thus, further studies to unveil the mechanisms that governs activation and release of IL-1β from VSMCs, and indeed other non-immune cells, are necessary to improve our knowledge of vascular biology and identify new potential therapeutic targets to treat cardiovascular diseases.

Study limitations

The results showed in this dissertation have some limitations. Firstly, the samples analysed were obtained from a relatively small number of subjects (4 individuals), therefore further studies in a larger group of patients have to be carried out to corroborate the conclusions. Second, the VSMCs utilised in this investigation were derived from human aortas, thus the mechanisms of synthesis, activation and release of IL-1β described previously could be significant in the development and/or prevention of aortic atherosclerotic plaques and aortic aneurysms. However, to investigate if mechanisms governing IL-1β biology are the same in coronary smooth muscle cells would be crucial, and would reveal the potential implications of these findings to coronary atherosclerosis - the main cause of ischemic heart disease and heart failure. Finally, the work presented in this thesis is based on *in vitro* assays and could not reflect what occurs *in vivo* due to changes of the protein expression of cells in culture and/or failure in capturing the complex interactions between cells and the surrounding environment.

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