Roles of ADAM10 and 17 in the shedding of LRP1 in human macrophages

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A thesis submitted for the degree of Doctor of Philosophy

University of East Anglia

MED

2024

Abstract

The inflammatory cytokine TNF has a central role in regulating the inflammatory response, and prolonged TNF release is observed in many chronic inflammatory diseases. The endocytic scavenger receptor LRP1 is involved in regulating TNF release from macrophages via the TIMP-3/ADAM17 axis. LRP1 can be shed from macrophages leading to an accumulation of TIMP-3, which inhibits ADAM17, and subsequently inhibits TNF release.

In this thesis I aimed to investigate how LRP1 was shed from human macrophages in response to inflammatory stimuli and which protease(s) were responsible for this shedding.

I first investigated LRP1 shedding in THP-1 and U937 human monocytic cell lines. However, as I was unable to detect shed LRP1 from U937 cells, and THP-1 cells were insensitive to LPS stimulation, I concluded that neither cell line was an appropriate model system for my investigations.

I next tested primary human monocyte-derived macrophages isolated from peripheral blood. Using these cells, I was able to detect constitutive LRP1 shedding after 2 hours of culture and LPS-stimulated LRP1 shedding after 6 hours of stimulation.

I used various protease inhibitors and blocking antibodies to target individual proteases to investigate which protease(s) were involved. I found that none of the broad specificity protease inhibitors significantly decreased LPS-stimulated LRP1 shedding and that a specific inhibitory blocking antibody against ADAM17 also had no effect. However, selective inhibition of ADAM10 inhibited both the constitutive and LPS-stimulated shedding of LRP1. Furthermore, I observed that activation of ADAM10 increased LRP1 shedding.

In conclusion, I have found that ADAM10 plays a key role in both the constitutive and stimulated shedding of LRP1 from human macrophages. Therefore, ADAM10 is involved in controlling the resolution of the inflammatory response by shedding LRP1 from macrophages to inhibit the continued release of TNF, which might otherwise result in chronic inflammation.

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List of Abbreviations

ACE-2	angiotensin-converting enzyme 2
ADAM	a disintegrin and metalloprotease
ADAMTS	a disintegrin and metalloprotease with thrombospondin motifs
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
ANOVA	analysis of variance
APOER	apolipoprotein E receptor
APP	amyloid precursor protein
ATCC	American type culture collection
BACE	β site of APP-cleaving enzyme
BRG1	Brahma-related gene 1
BSA	bovine serum albumin
CANDIS	conserved ADAM seventeen dynamic interaction sequence
CD	cluster of differentiation
cDNA	complementary DNA
cIAP1/2	cellular inhibitor of apoptosis 1/2
CRISPR	clustered regularly interspaced short palindromic repeats
Ct	cycle threshold
CX3CL3	chemokine (C-X3-C motif) ligand 1
DAMP	damage-associated molecular patterns
DLL	delta Like canonical Notch ligand
DMEM	Dulbecco’s modified eagle medium
DMSO	dimethyl sulfoxide

E-64	trans-epoxysuccinyl-l-leucylamido-(4-guanido)-butane
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EpCam	epithelial cell adhesion molecule
ER	endoplasmic reticulum
FBS	fetal bovine serum
FERM	four-point-one, erzin, radixin, moesin
FLT-3L	fms-related tyrosine kinase 3 ligand
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GH-R	growth hormone receptor
GP	glycoprotein
GST	glutathione-S-transferase
Hb-EGF	heparin binding EGF-like growth factor
HBSS	hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSPG	heparin sulfate proteoglycan
HRP	horseradish peroxidase
ICAM-1	intercellular Adhesion Molecule 1
ICOS-L	inducible co-stimulator ligand
IFN	interferon

IFT	intraflagellar transport
Ig	immunoglobulin
IGF-R	insulin-like growth factor receptor
IKK	IκB kinase
IL	interleukin
iPSC	induced pluripotent stem cell
IRF	interferon regulatory factor
iRHOM	inactive rhomboid protein
JAK/STAT	Janus kinase/signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase
KDM4	lysine demethylase 4
LAG-3	lymphocyte-activation gene 3
LDL-R	low-density lipoprotein receptor
LPS	lipopolysaccharide
LRP1	low-density lipoprotein receptor related protein 1
MAP	mitogen activated protein
M-CSF	macrophage colony-stimulating factor
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
MIC	MHC class I polypeptide–related sequence
MT1-MMP	membrane type 1 matrix metalloprotease
MMP	matrix metalloprotease
MRC	mannose receptor c-type

MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Muc-1	Mucin 1
NADPH	nicotinamide adenine dinucleotide phosphate
NEAA	non-essential amino acids
NHS	National Health Service
NLR	NOD-like receptor
PAGE	polyacrylamide electrophoresis gel
PAI-1	plasminogen activator inhibitor-1
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PenStrep	penicillin-streptomycin
PI3K	phosphoinositide 3-kinase
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethylsulfonyl fluoride
PPARγ	peroxisome proliferator-activated receptor-gamma
PRR	pattern-recognition receptor
PTP-LAR	protein tyrosine phosphatase of leukocyte antigen-related protein
PVDF	polyvinylidene difluoride
qRT-PCR	quantitative real time reverse transcription-polymerase chain reaction
RA	rheumatoid arthritis
RAP	receptor-associated protein
rcf	relative centrifugal force
RNA	ribonucleic acid

ROS	reactive oxygen species
RPMI	Roswell Park memorial institute
RSK	ribosomal S6 kinase
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulfate
Shc	src homology 2 domain-containing-transforming protein C
siRNA	small interfering RNA
Slit2	slit guidance ligand 2
SORCS	sortilin related vacuolar protein sorting 10 domain containing receptor
SORL	sortilin related receptor L
SORT	sortilin 1
SynCam	synaptic cell adhesion molecule
TACE	TNF-alpha converting enzyme
TAPI	TNF protease inhibitor
TCA	trichloroacetic acid
TGF	transforming growth factor
TIM	T-cell immunoglobulin and mucin
TIMP	tissue inhibitor of metalloprotease
TLR	toll-like receptor
TMEFF2	transmembrane protein with an EGF-like and two follistatin-like domains 2
TNF	tumour necrosis factor
TNFR	TNF receptor

tPA	tissue plasminogen activator
TRADD	TNFR1-associated death domain
TRAF2	TNFR-associated factor 2
TRANCE	TNF-related activation-induced cytokine
Tris	tris(hydroxymethyl)aminomethane
TSPAN	tetraspanin
TSPANC8	C8 family of tetraspanins
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
VEGF-R	vascular endothelial growth factor receptor

Acknowledgments

I would firstly like to thank my primary supervisor, Dr Linda Troeberg, for her endless support and guidance throughout my PhD, without which this thesis would not have been possible. Thanks to my secondary supervisor, Dr Jelena Gavrilovic for many helpful suggestions, comments, and ideas for future experiments.

I would also like to thank everyone in the Troeberg lab group for their constant help and support over these past three years. Thanks also to everyone in our Tuesday morning meetings for their interesting discussions and inputs from different points of view. I am incredibly grateful to everyone at the BCRE who made working in the lab so enjoyable and helped to teach me so many of the techniques used in this thesis.

Finally, I could not have done this without the unfailing support and encouragements of my family and friends.

Chapter 1: Introduction

1.1 Inflammation and chronic inflammatory disease

The acute inflammatory response is an essential step in the innate immune response to defend the body against harm from pathogens or tissue damage, and can be activated within minutes of detecting infection or injury. Acute inflammation is coordinated by a network of interconnected signalling pathways and ligands, and results in increased recruitment of immune cells and repair of tissue damage (Murphy & Weaver, 2016). The effects of the cytokines and other inflammatory mediators causes the symptoms of heat, pain, redness, and swelling that we associate with inflammation (Libby, 2007).

Initiation of the inflammatory response is mediated by the immune sensor cells, namely macrophages, neutrophils, and dendritic cells. These cells express a number of receptors called pattern-recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). PAMPs are evolutionarily-conserved molecular patterns expressed by pathogens but not by the host body's cells and can include lipopolysaccharides of the bacterial cell wall, mannose-rich oligosaccharides, and peptidoglycans (Zindel & Kubes, 2020). DAMPs are parts of the host cells which are not usually available to bind to the PRRs, but which are released and made available for binding upon cellular damage or stress (Frevert et al., 2018). Activation of PRRs on immune sensor cells directly stimulates the cells' phagocytic activity, and also stimulates their release of cytokines that further amplify the inflammatory response. PRRs are also expressed by non-immune cells such as endothelial cells (Janeway & Medzhitov, 2002), with activation of these upregulating various cytokines and other inflammatory mediators, and so stimulating leukocyte recruitment (Takeuchi & Akira, 2010).

PRRs can be subdivided into different groups, including toll-like receptors (TLRs) and NOD-like receptors (NLRs) (Kumar et al., 2011). Some TLRs are transmembrane proteins that recognise PAMPs originating from extracellular bacteria, and other TLRs that are intracellular are responsible for recognising signals such as viral genetic material (El-Zayat et al., 2019). There are a number of TLRs each with different ligands enabling the immune system to respond to a variety of signals. TLR4 is activated by gram-negative bacterial lipopolysaccharide (LPS) via a cluster of differentiation 14 (CD14)-dependent pathway, while TLR3 is activated by double stranded viral ribonucleic acid (RNA). TLR2 can form heterodimers with TLR1 and TLR6 to recognise ligands such as peptidoglycan and diacyl lipopeptides, and ligands of TLR5 include flagellin and microbial lipoproteins (Borish & Steinke, 2003). NLRs are cytoplasmic receptors responsible for sensing intracellular bacterial invasion.

A further consequence of initiation of the inflammatory response is an increase in vasodilation and vascular permeability, mediated by histamine (Medzhitov, 2008).

Circulating leukocytes are recruited and trafficked across the walls of the blood vessels to the site of inflammation via the leukocyte adhesion cascade (Ley et al., 2007), and there they phagocytose pathogens and damaged cells, and produce effector molecules such as reactive oxygen species (ROS), proteinase 3, and elastase (van den Bosch et al., 2020). These effector molecules are non-specific and can also target the body's own cells, resulting in off-target damage, that can be harmful to the host. This makes resolution of the inflammatory response a vital step ensuring that the pathogen/tissue damage is contained while minimising damage to the host (Feehan & Gilroy, 2019).

If the acute inflammatory response is not properly resolved (e.g., by a failure to remove a pathogen, development of an auto-immune response, or inability to terminate production of inflammatory mediators), then chronic inflammation can result (Nathan & Ding, 2010). Such chronic inflammation is implicated in a number of diseases including Crohn's disease (van Dullemen et al., 1995), colitis (Neurath et al., 1997), and rheumatoid arthritis (RA) (Feldmann, 2002).

RA is a systemic chronic inflammatory disease characterised by inflammation of articulating joints and hyperplasia of the synovial lining layer, leading to progressive joint damage and destruction. The resident cells within this layer are stimulated to upregulate the production of enzymes which degrade the cartilage and bone of the joint, while also increasing the production of cytokines promoting immune cell infiltration (Falconer et al., 2018). The eventual result of these processes is destruction of the joint. Dysregulation of innate immunity is observed, including immune complex-mediated complement activation, an adaptive immune response to self-antigens, and dysregulated cytokine signalling pathways (Firestein & McInnes, 2017). The precise etiology of RA is complex with many different components including genetic and environmental factors.

Crohn's disease is characterised by chronic inflammation of the gastrointestinal tract and eventually can lead to stricture or penetrating lesions requiring surgery. The initial insult that causes Crohn's disease is unclear and the pathophysiology is complex and dependent on the interplay of a variety of factors (Torres et al., 2017). These factors include genetic susceptibility, environmental factors, and changes in the intestinal microbiota, which combine to produce an abnormal mucosal immune response.

While both of these chronic inflammatory conditions have complex causes, they share chronic activation of the immune system and persistent inflammation. Chronic inflammation has been shown to be controlled by inflammatory mediators and cytokines produced by leukocytes such as tumour necrosis factor (TNF), interleukin-6 (IL-6), and IL-17 (Noack & Miossec, 2017). TNF in particular has been shown to be the central cytokine in RA pathophysiology responsible for regulating the production of the other cytokines (Brennan

& McInnes, 2008; Kalliolias & Ivashkiv, 2016; McInnes & Schett, 2007). This has led to the development of anti-TNF therapies for first RA, and then other chronic inflammatory diseases (Feldmann, 2002; Feldmann et al., 1995; van Dullemen et al., 1995). The efficacy of anti-TNF therapies in treating multiple chronic inflammatory diseases reflects the common mechanism which they share, and shows the importance of TNF in chronic inflammation as well as the importance of proper regulation of the innate immune response and inflammation.

1.1.1 Roles of macrophages in the inflammatory response

Macrophages are a key part of the innate immune system and have a number of functions that are vital for the coordination of the immune response. Along with other phagocytic mononuclear cells, they are the first line of defence in protection against pathogens and tissue injury. They were first described as cells able to phagocytose foreign particles by Elie Metchnikoff (Gordon, 2016) but have since been shown to orchestrate the immune response by activating and regulating other immune cells, via presentation of antigens and secretion of inflammatory mediators such as cytokines and chemokines (Fujiwara & Kobayashi, 2005). Macrophages are resident in every tissue of the body, allowing for rapid detection of infection or injury wherever it occurs. These tissue-resident macrophages are created during embryonic development and are extremely heterogeneous. In addition to their immune-surveillance and immune-regulatory functions, they also help to maintain homeostasis of their specific tissue niche by clearing cellular debris and regulating iron availability (Davies et al., 2013).

There is also a circulating population of monocytes that originate from the bone marrow and make up 4-10% of nucleated cells in the blood (Coillard & Segura, 2019; Shi & Pamer, 2011). In response to chemokines, these cells can migrate into tissues and differentiate there into macrophages. Upon activation of the inflammatory response, the population of monocytes can be rapidly expanded and then migrate to the site of inflammation where they can differentiate to macrophages to help the tissue-resident population (Murray & Wynn, 2011).

Once monocytes have differentiated into macrophages at the site of inflammation, they undergo a process of polarisation that determines their phenotype (Mosser & Edwards, 2008). This process is controlled by a number of both extrinsic and non-extrinsic factors, including inflammatory cytokines and PAMPs/DAMPs, the tissue microenvironment in which the macrophage has migrated to, and the cell survival pathways within the cell (Murray, 2017), and results in a complex spectrum of polarisation states.

Historically, macrophage polarisation was considered to involve two distinct outcomes, ‘M1’ or pro-inflammatory macrophages, and ‘M2’ or anti-inflammatory, regulatory macrophages (Martinez & Gordon, 2014). Macrophages were shown to polarise to an M1-like state in response to pro-inflammatory cytokines such as interferon (IFN)-γ (Shapouri-Moghaddam et al., 2018) or by TLR/NLR agonists, TNF, and IL-1 (Kratochvill et al., 2015; Murray, 2017). These pro-inflammatory cells secrete pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, TNF, and IFN-γ that amplify inflammation and can activate the adaptive immune response by presenting antigens (Mills & Ley, 2014). They also have high ability to phagocytose both microorganisms and cell debris (Atri et al., 2018). They are often characterised by the expression of the cell surface markers CD80 and CD64 (Tarique et al., 2015).

On the other hand, macrophages can polarise to an M2-like state. This state of polarisation was identified as stimulated by IL-4 and IL-13 produced by eosinophils and basophils in response to helminth infection (Loke et al., 2002), however there are a number of other conditions which favour M2-like polarisation (Martinez & Gordon, 2014; Verreck et al., 2006), including production of IL-10 by leukocytes. Macrophages polarised in this manner have an immune-modulatory activity, and express reduced levels of pro-inflammatory cytokines and increased levels of anti-inflammatory mediators such as transforming growth factor-β (TGF-β), IL-4, and IL-10 (Jaguin et al., 2013; Schraufstatter et al., 2012). There have been a number of subcategories proposed for M2-like macrophages (Martinez & Gordon, 2014). M2a macrophages were defined as produced in response to IL-4 and IL-13, with M2b macrophages generated in response to immune complexes, and M2c in response to glucocorticoids or IL-10 (Juhas et al., 2015). These subsets vary in cytokine secretion and marker expression, demonstrating some of the complexity of how macrophages can respond to their environment.

The terms ‘M1’ and ‘M2’ are controversial and considered outdated, due to the lack of defined characteristics for each phenotype (Murray et al., 2014) and a large body of literature showing that macrophage polarisation in vivo is not binary, but rather highly variable, leading to a spectrum of heterogenous phenotypes (Avraham et al., 2015; Junkin et al., 2016; Shalek et al., 2013). In addition, polarisation has been shown to be reversible and macrophages can be re-polarised from a pro-inflammatory phenotype to an anti-inflammatory one, and vice versa (Xu et al., 2013). This adds a further layer of complexity to the model of macrophage polarisation. Despite this, the paradigm of ‘M1’ and ‘M2’ still retains some use as representing the two extremes at either end of the continuum of macrophage polarisation.

Dynamic polarisation of macrophages in response to their environment is key to a regulated inflammatory response. When tissue-resident macrophages sense infection or injury via PRRs, they polarise into an inflammatory state, enabling recruitment of circulating

monocytes from the blood and their differentiation into macrophages that polarise in response to the local microenvironment. Throughout the inflammatory process, the tissue microenvironment changes, altering the stimuli that macrophages are exposed to and so altering the polarisation state of the macrophages in a highly dynamic manner (Varga et al., 2016) to achieve co-ordinated progression from inflammation to resolution.

During the early, acute phase of inflammation, macrophages exhibit a more pro-inflammatory phenotype, expressing and secreting pro-inflammatory cytokines such as TNF which further upregulates the inflammatory response (Mantovani et al., 2004). As tissue repair progresses, macrophage phenotype becomes less inflammatory, and the cells start secreting anti-inflammatory mediators and cytokines that inhibit the effects of pro-inflammatory macrophages and accelerate wound healing and tissue repair by promoting myofibroblast differentiation and angiogenesis along with collagen deposition (Arnold et al., 2007; Italiani et al., 2014). This temporal switch from pro-inflammatory to anti-inflammatory macrophages has been observed across many different types of infection and tissue injury (Pearce & MacDonald, 2002).

Many chronic diseases with an inflammatory component (e.g., atherosclerosis) have been associated with dysregulation of macrophage polarisation (Bashir et al. 2016). Genetic variants of interferon regulatory factor 5 (IRF5), a transcription factor that contributes to the pro-inflammatory macrophage phenotype (Krausgruber et al., 2011), have been associated with RA (Dieguez-Gonzalez et al., 2008) demonstrating that disrupted macrophage polarisation increases susceptibility to chronic inflammatory conditions.

Tight regulation of TNF release from macrophages is very important for overall regulation of the inflammatory response. TNF is considered to be the apex pro-inflammatory cytokine, as it induces the expression of the other pro-inflammatory cytokines in an inflammatory cascade that is characteristic of the acute inflammatory response (Feldmann, 2002; Murphy & Weaver, 2016). Dysregulated TNF release thus leads to chronic inflammation, with elevated and prolonged TNF release associated with chronic inflammatory conditions like RA (Feldmann et al., 1999), Crohn's disease (van Dullemen et al., 1995), colitis (Neurath et al., 1997), and psoriasis (Ko et al., 2009).

Both the transmembrane and soluble forms of TNF can signal through binding to either of its receptors; TNF receptor 1 (TNFR1), which is ubiquitously expressed in all cell types, or TNFR2, which is primarily expressed in immune cells, neurons, and endothelial cells (Faustman & Davis, 2010). Membrane bound TNF predominantly binds to TNFR2 and acts to transmit cell-cell interactions (Grell et al., 1995) and can also act as a receptor itself through the initiation of intracellular signalling cascades (Eissner et al., 2004). Although the extracellular regions containing the TNF binding sites of TNFR1 and TNFR2 are similar, the

intracellular regions are distinct, leading to the recruitment of different adaptor proteins as a result of stimulation (Tartaglia et al., 1991). Binding of TNF to either TNFR1 or TNFR2 can result in the activation of the NF-κB pathway, leading to an activation of the inflammatory response and the induction of a cell survival response (Figure 1.1). TNFR1 signalling can also produce a cell death response depending on other cell death checkpoints which further regulate the response of the cell to TNF (Ting & Bertrand, 2016). TNF signalling in this manner is tightly controlled through post-translational polyubiquitination of various proteins within the pathway (Y. Wu et al., 2018). These chains are assembled in response to activation of the proteins through phosphorylation or conformational change and depending on the specific linkage type used, can have a variety of effects on the target protein. For example, the addition of the polyubiquitin chain to RIP1 activates it, leading to the recruitment of the IKK complex (Haas et al., 2009). Conversely, ubiquitination of IκBα, further downstream in the pathway, marks it for degradation by the proteosome, therefore releasing the NF-κB transcription factor to the nucleus.

While TNF, TNFR1, and TNFR2 are the best characterised and understood members of the TNF superfamily, in total there are 19 ligands and 29 receptors that comprise the wider TNF superfamily (Wallach, 2018). Several of the ligands can bind to multiple receptors while some receptors can bind more than one ligand. This gives rise to an extensive shared network of signalling which is involved in the regulation of immunity and homeostasis. The majority of the TNF superfamily members are expressed in immune cells and their expression can be induced by the activation of the immune response. They are involved in the maintenance, survival, and homeostasis of lymphocytes and leukocytes and so consequently are considered major factors in the development of inflammatory diseases (Dostert et al., 2019). The ligands and receptors vary in their expression profiles across different cell types; however, TNF and TNFR1/2 are the predominant members of the superfamily expressed in macrophages and are primarily responsible for the control of the inflammatory response (Holbrook et al., 2019).

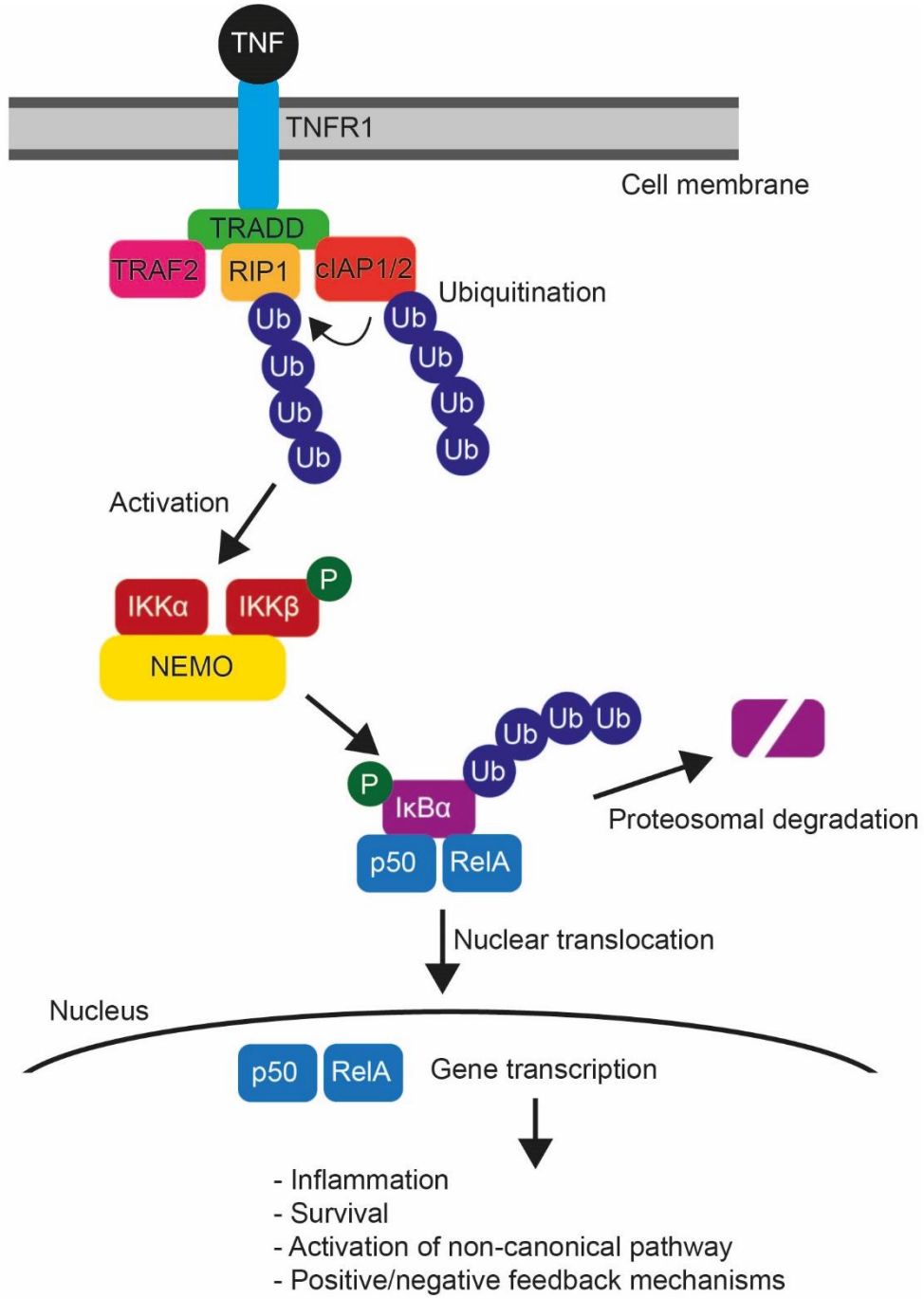

Figure 1.1 TNF can signal through the NF-κB pathway to activate the inflammatory response.

When TNF binds to TNFR1, TNFR1-associated death domain (TRADD) is recruited which then subsequently binds to TNFR-associated factor 2 (TRAF2), receptor-interacting serine/threonine-protein kinase 1, and cellular inhibitor of apoptosis 1 or 2 (cIAP1/2). This results in the sequential polyubiquitination of cIAP1/2 followed by RIP1. This stabilises RIP1 leading to the amplification of its signal which results in the recruitment and phosphorylation of the IκB kinase (IKK) complex. IκBα is subsequently polyubiquitinated and degraded by the proteosome releasing the transcription factor NF-κB which consists of p50 and RelA. NF-κB translocates to the nucleus leading to the transcription of target genes resulting in the activation of the inflammatory response.

TNF is synthesised as a transmembrane precursor, pro-TNF, that is cleaved by the type 1 transmembrane metalloprotease a disintegrin and metalloprotease 17 (ADAM17) to generate a soluble form that is released or “shed” from cells (Black et al., 1997).

1.2 ADAMs and the release of TNF

The ‘a disintegrin and metalloprotease’ (ADAM) family of trans-membrane metalloproteases are part of the adamalysin protease family. There are 24 *ADAM* genes in humans, of which 4 are pseudogenes, 12 encode catalytically active ADAM metalloproteases, and 8 encode inactive proteases that lack catalytic activity (Klein & Bischoff, 2011). The physiological roles of the inactive human ADAMs have not been extensively studied and are largely unknown. Some have been shown to act as adhesion molecules rather than proteases and play roles in development (Blobel et al., 1992). The active ADAMs have been much more extensively studied.

Most of them are broadly expressed across many cell types although *ADAM20, 21*, and *30* are only expressed in the testis (Edwards et al., 2009). They contain a characteristic conserved Zn^{2+}-binding motif (HEXGHXXGXXHD) in their active site that is necessary for catalysis, with the 3 histidine residues coordinating a Zn^{2+} ion that drives substrate hydrolysis (Bode et al., 1993). This motif is also present in the other members of the metzincin superfamily, such as matrix metalloproteases (MMPs) and ‘a disintegrin and metalloprotease with thrombospondin motifs’ (ADAMTSs) (Nagase et al., 2006), but is mutated and does not bind Zn^{2+} in the inactive ADAMs.

ADAMs are composed of approximately 750 amino acids, that can be divided into a number of functional domains (Figure 1.2). A signal peptide at the N-terminus directs the ADAMs to the secretory pathway (Edwards et al., 2009), and this is followed by a chaperone-like prodomain that ensures correct protein folding and is generally removed intracellularly in the Golgi by a pro-protein convertase such as furin (Srour et al., 2003). In ADAM8 and 28, the prodomain is removed by autocatalysis as they lack a recognition site for furin cleavage (Howard et al., 2000; Schlomann et al., 2002). The prodomain also prevents activation of the ADAMs intracellularly and its removal exposes the catalytic domain, which contains the active site. This is followed by the disintegrin domain, cysteine-rich domain containing a hypervariable region, and the membrane-proximal stalk region. In the majority of the proteolytically active human ADAMs, this stalk region is an epidermal growth factor (EGF)-like repeat domain, but in ADAM10 and 17 this domain is absent. ADAM17 contains a short motif termed the ‘conserved ADAM seventeen dynamic interaction sequence’ (CANDIS) in the stalk region, which interacts with the cell membrane (Düsterhöft et al., 2014). This is followed by the transmembrane domain and the cytoplasmic tail. The C-terminal

cytoplasmic tails of the ADAMs are highly variable in length and sequence (Takeda, 2016), and ADAM9, 12, and 28 have splice variants that lack both the transmembrane and cytoplasmic domains, and are therefore expressed as soluble active proteases (Mazzocca et al., 2005; Wewer et al., 2006).

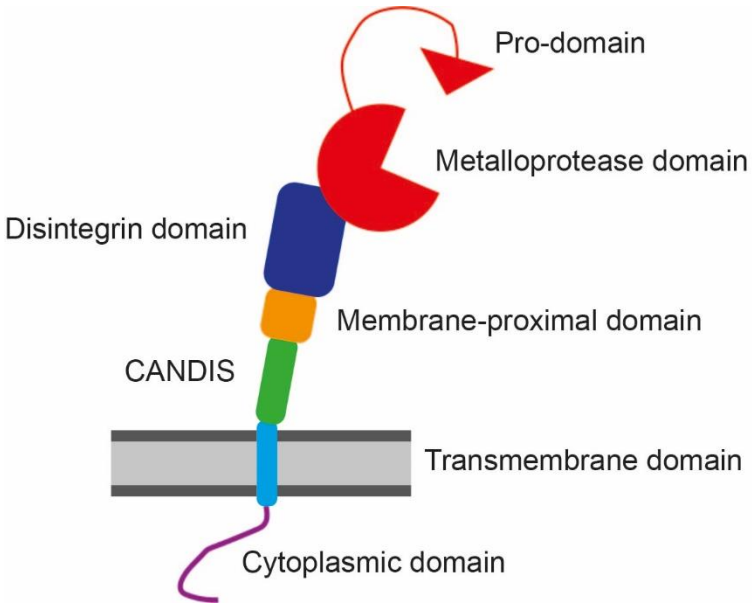

Figure 1.2 Schematic of ADAM17 domain structure.
ADAM17 is composed of seven domains, one of which, the pro-domain, is removed in the Golgi by the pro-protein convertase furin. Removal of the pro-domain is required to expose the active site within the metalloprotease domain. This domain contains the Zn^{2+}-binding motif necessary for substrate hydrolysis. The membrane-proximal domain and CANDIS are involved in the regulation of ADAM17 activity by forming electrostatic interactions with the cell membrane and altering the conformation of ADAM17.

The most studied function of the ADAM metalloproteases is as “sheddases” of other transmembrane proteins by proteolytic cleavage. This was first demonstrated in 1997 by two separate groups who identified ADAM17 as the protease responsible for cleaving pro-TNF to release soluble TNF (Black et al., 1997; Moss et al., 1997). For this reason, ADAM17 is also known as TNF-alpha converting enzyme (TACE). Through the shedding of their substrates, ADAMs can influence paracrine, autocrine, and juxtacrine signalling events. Due to the very broad range of substrates they can target, ADAMs play a major role in many signalling pathways and cellular processes, ranging from cell proliferation (Edwards et al., 2009), to immune signalling (Doedens et al., 2003) and cancer progression (Düsterhöft et al., 2019; Wang et al., 2022).

1.2.1 ADAM17 plays a role in many signalling pathways through shedding a diverse range of substrates

ADAM17 is the most extensively studied of the ADAMs, as it cleaves a wide range of substrates involved in a number of important cellular processes (Table 1.1) (Calligaris et al., 2021; K. Wang et al., 2022; Zunke & Rose-John, 2017). ADAM17 substrates include signalling molecules and their receptors, cytokines, growth factors, and adhesion molecules.

Table 1.1 Substrates cleaved by ADAM17.

ADAM17 has been shown to cleave more than 90 substrates, with roles in a variety of different cellular processes, including development, cell adhesion, and immune function (Calligaris et al., 2021; K. Wang et al., 2022; Zunke & Rose-John, 2017).

Immune system	Development	Cell adhesion	Others
APOER	Amphiregulin	CD44	ACE-2
CD16	CD36	CD166	APP
CD163	DLL1	Collagen XVII	APP-like protein2
CD89	Epigen	Desmoglein 2	Carbonic hydrolase 9
CSF-1	ErBB4	EpCam	Ebola virus glycoprotein
CX3CL1	Epiregulin	ICAM-1	Endothelial protein C receptor
ICOS-L	FLT-3L	Junctional Adhesion Molecule A	GPIba
IL-6R	GH-R	L1 Cell Adhesion Molecule	GPV
IL-15R	Hb-EGF	L-selectin	GPVI
IL-1R2	IGF2-R	Neural Cell Adhesion Molecule	Klotho
LAG-3	Jagged	Nectin-4	Muc-1
LDL-R	LY6/PLAUR Domain Containing 3	SynCAM1	Natriuretic peptide receptor
Lymphotoxin α	Notch1	Vascular cell adhesion protein 1	Pre-adipocyte factor
M-CSFR	Neuregulin 1		Prion protein
MIC-A	Premelanosome 17		
MIC-B	PTP-LAR		
SORCS1			

SORCS3 SORL1 SORT1 TIM-1 TIM-3 TIM-4 TNF TNF-R1 TNF-R2 TNFRSF5 TNFRSF8 TRANCE	Semaphorin 4D Syndecan1 Syndecan4 TMEFF2 TGFα Tropomyosin receptor kinase A Vasorin VEGF-R2		Protein Tyrosine Phosphatase Receptor Type Z1

ADAM17 is ubiquitously expressed, although highest expression is observed in the lung placenta, and testis. ADAM17-deficient mice are not viable and die shortly after birth due to dysregulated epidermal growth factor receptor (EGF-R) signalling (Peschon et al., 1998) which has made knock-out studies difficult. In order to solve this issue, a number of strategies have been developed including generation of conditional knockout mice lines. Three of the most physiologically important ADAM17 substrates (i.e. TNF, IL-6R, and EGF-R) have been extensively studied using these methods.

TNF is expressed as a transmembrane precursor (called pro-TNF), which is then cleaved by ADAM17 to release soluble TNF from cells (Black et al., 1997). As previously described (Section 1.1.1) TNF release plays a major role in regulation of inflammation.

Horiuchi *et al.* (2007) generated conditional ADAM17-deficient mice which lacked ADAM17 in myeloid cells, and also used the Mx1-Cre model for temporal inactivation of ADAM17 in the hematopoietic stem cell lineage. Both groups of mice were protected against endotoxin shock lethality resulting from injection of LPS, as lack of ADAM17 prevented the lethal increase in serum TNF levels.

These findings were further supported by generation of a viable mouse line with barely detectable levels of ADAM17 in all cell types (Chalaris et al., 2010). Generation of soluble TNF was almost completely inhibited in the resultant ADAM17$^{ex/ex}$ hypomorphic mice. These studies demonstrated that ADAM17 is the primary endotoxin-stimulated sheddase of TNF

in murine myeloid cells in vivo, defining a central role for ADAM17 in TNF release and regulation of inflammation.

ADAM17 is also responsible for the shedding of TNF receptors, as chimeric mice lacking leukocyte expression of *ADAM17* had greatly reduced levels of soluble TNF, TNFR1, and TNFR2 in response to induction of inflammation (Bell et al., 2007). Both receptors bind to soluble TNF, but TNFRII is primarily activated by membrane bound pro-TNF (Grell et al., 1995). Shedding of TNF receptors acts as a negative feedback mechanism, as it reduces cellular capacity for TNF signalling, and the soluble receptors can act as decoy receptors that bind TNF and prevent it from interacting with cell surface signalling receptors (Kalliolias & Ivashkiv, 2016).

By controlling the shedding of both TNF and TNF receptors, ADAM17 plays a central role in temporal regulation of TNF signalling.

Another well-studied substrate of ADAM17 is IL-6R, which is a type 1 transmembrane receptor for the cytokine IL-6 (Yamasaki et al., 1988). IL-6 binding to a complex of the IL-6R and glycoprotein130 (gp130) initiates signal transduction by promoting dimerization of the IL-6/IL-6R/gp130 receptor complex and subsequent activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and phosphoinositide 3-kinase (PI3K) pathways (Hibi et al., 1990) (Figure 1.3). This classical IL-6 signalling results in resolution of inflammation (Heinrich et al., 1998), but the IL-6R is only expressed in hepatocytes and lymphocytes, so this pathway only takes place in a limited number of cells. However, it was observed that the IL-6 receptor can be cleaved and shed from the cell membrane as a soluble receptor in response to phorbol ester stimulation (Müllberg et al., 1993). This soluble form of the IL-6R was still able to bind to IL-6 and the complex is able to bind to gp130 receptors (Mackiewicz et al., 1992). This enables IL-6 signalling to take place in cells that do not express IL-6R, as gp130 is ubiquitously expressed. Such IL-6 trans-signalling has the opposite effect to classical IL-6 signalling as it upregulates immune activation and has been associated with RA and other chronic inflammatory conditions (Calabrese & Rose-John, 2014).

Regulation of IL-6R shedding is thus another important mechanism controlling the immune response. In vitro, the IL-6R can be cleaved by ADAM17 and ADAM10 (Matthews et al., 2003). In vivo however, using a conditional knockout of *Adam17* in mice, it was shown that the rapid shedding of IL-6R in response to inflammatory stimuli was the result of ADAM17 activity alone (Yan et al., 2016). These studies thus identify ADAM17 as the primary physiological sheddase of IL-6R, extending our understanding of the protease's central role in regulating key signalling pathways of the inflammatory and acute immune response.

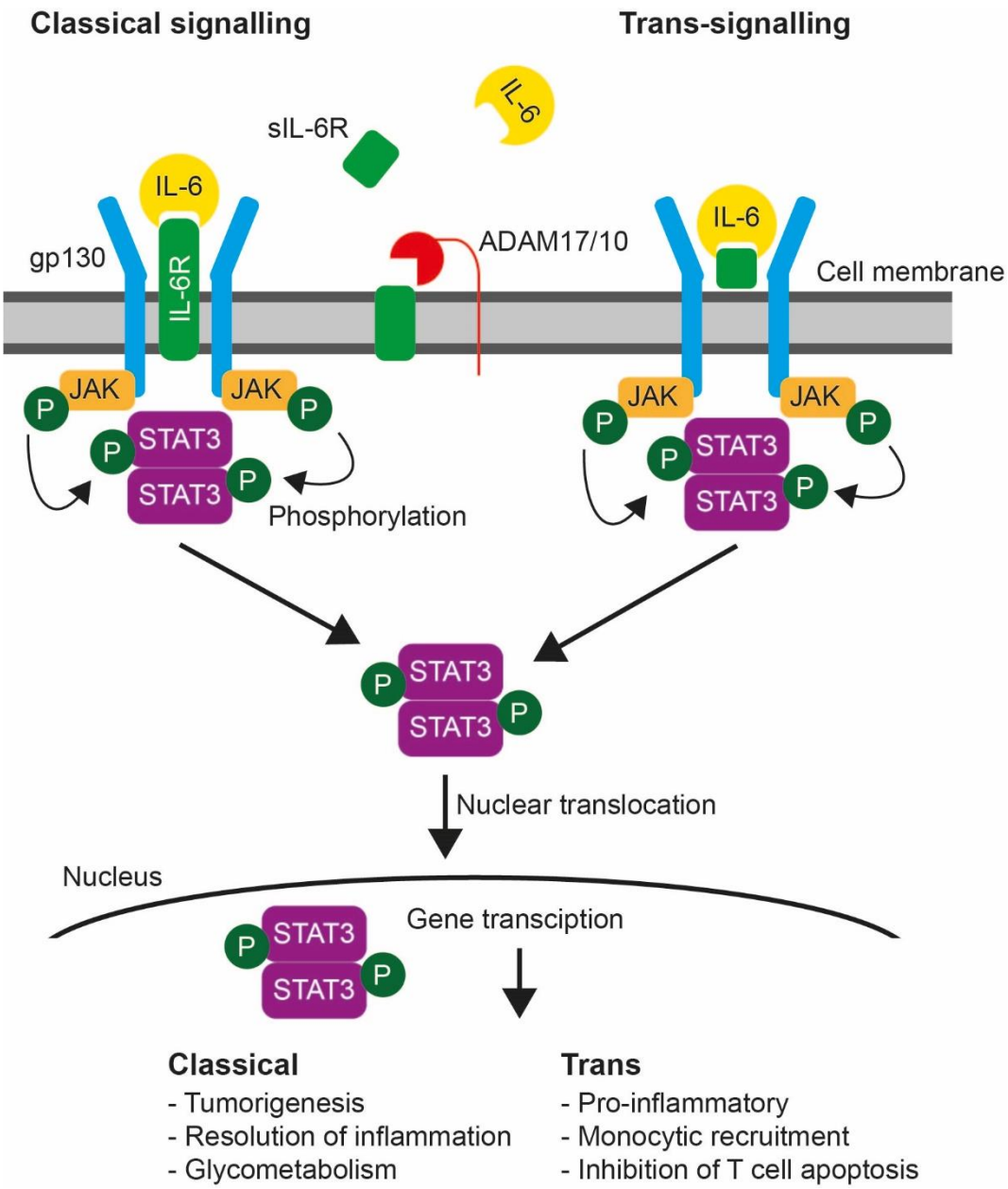

Figure 1.3 IL-6 classical or trans-signalling.

In classical signalling, IL-6 binds to the membrane bound IL-6R resulting in the recruitment and dimerization of gp130. This leads to phosphorylation and activation of the tyrosine kinase JAK which in turn phosphorylates the transcription factor STAT3. In trans-signalling, IL-6 binds to the soluble form of the IL-6R which has been cleaved and shed by ADAM10 or ADAM17. IL-6/sIL-6R can then bind, recruit, and dimerize gp130 resulting in downstream signalling through the JAK/STAT pathway. The two different pathways for IL-6 signalling result in opposite effects on the inflammatory response.

ADAM17 also plays a vital role in cellular signalling by shedding the ligands of the EGF-R. However, in this case, the signalling pathways are involved in development rather than inflammation, demonstrating the broad range of processes in which ADAM17 is involved. There are many ligands involved in EGF-R signalling and these are synthesised as

transmembrane precursors, which must be proteolytically cleaved and shed as a pre-requisite to signalling (Blobel et al., 2009). Both ADAM17 and ADAM10 shed EGF-R ligands, with ADAM17 shown to be responsible for shedding of TGF-α, epiregulin, epigen, amphiregulin, and heparin-binding EGF-like growth factor (Sahin et al., 2004; Sahin & Blobel, 2007).

The murine lethality of the constitutive *Adam17* knockout is primarily due to the lack of EGF-R signalling (Peschon et al., 1998). These mice are born with open eyes and die perinatally, and this phenotype is replicated by constitutive knockout of *Tgfα*. Peschon *et al.* (1998) thus provided key evidence to support the role of ADAM17 in TGF-α shedding and demonstrated the importance of ADAM17-mediated EGF-R signalling during development. Subsequent studies using the *Adam17* conditional knockout mice have supported these findings. For example, ADAM17-dependent EGF-R signalling was shown to be required for skeletal development, with deletion of *ADAM17* in chondrocytes leading to an accumulation of hypertrophic chondrocytes, and retarded growth of the long bones (Hall et al., 2013). In vitro studies show that hypertrophic differentiation of chondrocytes is increased by deletion of *ADAM17* or *EGF-R* (Wang et al., 2004), indicating that ADAM17 promotes growth of long bones by facilitating EGF-R signalling. Similarly, ADAM17-dependent amphiregulin signalling was shown to be required for mammary gland development (Sternlicht et al., 2005) and ADAM17-dependent TGF-α signalling was shown to promote epidermal barrier function, with conditional deletion of *Adam17* in keratinocytes causing defects in epidermal barrier integrity soon after birth and chronic dermatitis in adult mice (Franzke et al., 2012). This phenotype could be rescued with the treatment of TGF-α, demonstrating that it is caused by the lack of soluble TGF-α production by ADAM17.

These in vivo studies demonstrate that ADAM17-mediated shedding plays a crucial role in TNF-, IL-6-, and EGF-R-dependent signalling pathways and hence in a wide variety of physiological contexts. It is thus vitally important that the activity of ADAM17 is tightly controlled.

1.2.2 Molecular mechanisms regulating ADAM17 activity

ADAM17 exhibits low levels of basal catalytic activity on the cell surface, until its activity is activated in response to physiologically relevant [e.g. LPS and G-protein coupled receptors (Arora et al., 2008)] or experimental [e.g. phorbol-12-myristate-13-acetate (PMA), a phorbol ester that induces phosphorylation of protein kinase C (le Gall et al., 2010)] stimuli. Such stimuli allow for rapid and reversible activation of substrate cleavage, often without any increase in cell surface levels of expression (Horiuchi et al., 2007; Killock & Ivetić, 2010; Willems et al., 2010). As discussed below, molecular mechanisms regulating ADAM17 activity include its trafficking, conformation, and interaction with inactive rhomboid proteins

(iRHOMs), as well as substrate localisation and structure, and ADAM17 interaction with its endogenous inhibitor tissue inhibitor of metalloprotease-3 (TIMP-3).

Transcriptional regulation of *ADAM17* has been described and is likely to be of importance in chronic inflammatory diseases including RA, atherosclerosis, and some cancers (Ringel et al., 2006). *ADAM17* expression can be stimulated by transcription factors such as the p65 subunit of NF-κB and the Elk-1 transcription factor which is activated downstream of ERK1/2 signalling pathway (Wawro et al., 2019). Inflammatory stimuli and phorbol esters that activate ADAM17 at the cell surface can thus also upregulate *ADAM17* expression to further increase shedding activity over a longer time period.

Transcription of *ADAM17* can also be affected by epigenetic factors. For example, in response to Wnt signalling, β-catenin binding to the *ADAM17* promoter can recruit the chromatin-remodelling protein Brahma-related gene 1 (BRG1) and subsequently the histone demethylase lysine demethylase 4 (KDM4), resulting in transactivation of *ADAM17* expression by alteration of chromatin structure (Sun et al., 2020). In addition, a number of micro-RNAs have been demonstrated to downregulate *ADAM17* expression e.g. miR-145 binds directly to the *ADAM17* 3'-UTR and suppresses expression (Lu et al., 2012; Yang et al., 2014).

The pro-domain of ADAM17 plays an important role in regulating the catalytic activity during ADAM17 synthesis via the secretory pathway (Buckley et al., 2005). This prevents ADAM17 from cleaving proteins intracellularly during its maturation. The pro-domain is only removed once ADAM17 has been trafficked into the Golgi from the endoplasmic reticulum (ER). This cleavage is carried out by the pro-protein convertase furin, which processes the pro-domain at two sites resulting in the mature, active form of ADAM17 which can then be trafficked to the cell membrane (Wong et al., 2015). A point mutation in the furin processing site of the pro-domain has been observed in colon cancer patients, and found to prevent maturation of ADAM17 and interfere with its trafficking (Pavlenko et al., 2019). The majority of ADAM17 is stored intracellularly, rather than on the cell surface, and there is a constitutive level of turnover that maintains a constant level of ADAM17 on the cell surface under basal conditions (Doedens & Black, 2000; Dombernowsky et al., 2015; Soond et al., 2005). ADAM17 can be internalised in a clathrin-dependent manner, and then either recycled to the cell surface or degraded via the lysosomal pathway (Lorenzen et al., 2016).

One of the major mechanisms by which ADAM17 activity is regulated is through its interactions with the inactive rhomboid proteins (iRHOMs). iRHOM1 and iRHOM2 are seven-membrane-spanning inactive proteases in the rhomboid family that were discovered to play an essential role in the maturation, trafficking, and activity of ADAM17 (McIlwain et al., 2012).

iRHOMs consist of 3 main structural subunits: (i) the seven-transmembrane domains consist of 6 transmembrane helices separated from a final helix by a rhomboid fold (Lemberg & Freeman, 2007). This subunit is common across the rhomboid superfamily. (ii) a large N-terminal cytosolic tail (≈ 400 amino acids in length) that makes up around half of the protein, and (iii) an iRHOM homology domain located extracellularly between the first and second transmembrane domains (Dulloo et al., 2019). The latter 2 regions are unique to iRHOMs.

Most cells express both *iRHOM1* and *iRHOM2,* except for myeloid cells, where expression of *IRHOM1* is very low, and so iRHOM2 is the primary regulator of ADAM17 (Issuree et al., 2013). As a result, iRHOM2 is an important regulator of TNF release from macrophages and so of inflammation.

iRhom2 knockout mice have very similar phenotypes to myeloid-specific *Adam17* conditional knockout mice, and are protected from endotoxin shock (McIlwain et al., 2012). The lack of iRHOM2 has little effect on most other cell types, indicating that iRHOM1 can largely substitute for iRHOM2 (Li et al., 2015). The phenotype of *iRhom1* knockout mice appears to vary substantially with genotype, with knockout on a C57BL/6N background showing no phenotype (Li et al., 2015), but knockout on a C57BL/6J and mixed 129S6/SvFvTac strains leading to severe weight loss after birth and pronounced intracerebral haemorrhage (Christova et al., 2013). This indicates that in some cases iRHOM2 cannot substitute for iRHOM1. *iRhom1/2* double knockout mice exhibit severe phenotypes, albeit with similar strain-dependent variation. *iRhom1/2* double knockout mice generated by Li *et al.* (2015) reproduce the phenotype of *Adam17*-deficient mice in that they are born with open eyes and die perinatally, while mice generated by Christova *et al.* (2013) showed a much more severe phenotype with embryonic lethality. This demonstrates the important role of iRHOMs in regulating ADAM17 activity, and hence EGF-R signalling, during development.

The iRHOM/ADAM17 complex remains together throughout the maturation and trafficking of ADAM17 and iRHOMs are now considered an essential part of the ADAM17 proteolytic complex (Figure 1.4). iRHOMs regulate ADAM17 activity by several mechanisms. They are essential for the maturation of ADAM17 and its trafficking through the secretory pathway to the cell membrane (Adrain et al., 2012). They are also involved the stability of ADAM17 at the cell surface and are important for maintaining its cell surface levels. Disrupting the iRHOM/ADAM17 complex results in increased endocytosis and lysosomal degradation of the mature ADAM17 and the iRHOM (Grieve et al., 2017). Binding of the four-point-one, erzin, radixin, moesin (FERM) domain-containing protein FRMD8 to the cytoplasmic N-terminal tail of iRHOMs is necessary for the prevention of endolysosomal degradation of ADAM17 (Künzel et al., 2018; Oikonomidi et al., 2018), and this relationship is reciprocal,

with ADAM17 also stabilising iRHOM2 and preventing its degradation (Weskamp et al., 2020).

IRHOMs can modulate ADAM17 stimulation at the cell surface. The N-terminal cytoplasmic tail of iRHOMs can be phosphorylated at numerous sites in response to ADAM17 stimuli such as LPS and PMA (Cavadas et al., 2017; Grieve et al., 2017). Phosphorylation is dependent on mitogen activated protein (MAP) kinases [p38α, ERK1, ERK2, ribosomal S6 kinase 1 (RSK1), and RSK2] stimulated downstream of TLR activation by LPS (Díaz-Rodríguez et al., 2002; Yamamoto et al., 2002) and is required for the rapid stimulation of ADAM17 activity but not its maturation or trafficking.

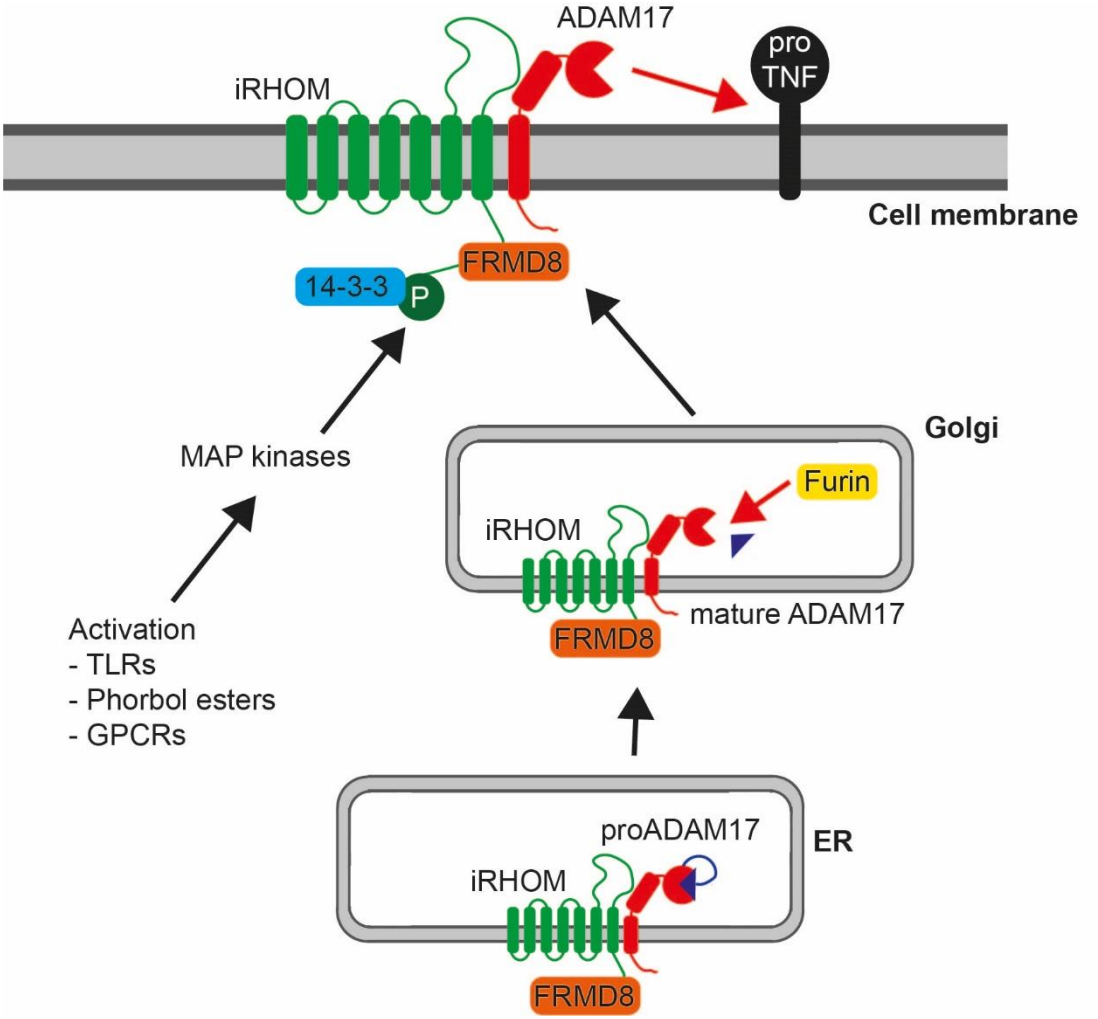

Figure 1.4 Regulation of ADAM17 by the iRHOMs.

The interaction between ADAM17 and an iRHOM is required for trafficking from the ER to the Golgi so that furin-mediated maturation can take place. The iRHOM/ADAM17 complex remains together at the cell membrane and the binding of FRMD8 to the cytoplasmic domain of iRHOM1/2 is necessary to prevent the endolysosomal degradation of ADAM17. ADAM17 activating stimuli such as LPS or PMA stimulate MAP kinases that phosphorylate the cytoplasmic tail of iRHOM1/2, resulting in the recruitment of 14-3-3 proteins and subsequent

ADAM17 activation. The iRHOM which is in complex with ADAM17 at the cell membrane may be involved in substrate selectivity.

Of the numerous sites on the cytoplasmic tail of iRHOM2 that can be phosphorylated, phosphorylation at Ser83 is most important for ADAM17 stimulation, as this was only the site where the absence of phosphorylation significantly impaired shedding (Cavadas et al., 2017). 2 other phosphorylation sites (Ser60 and Ser359) have also been shown to have roles in ADAM17 stimulation (Grieve et al., 2017).

Phosphorylation of the iRHOM2 cytoplasmic tail enables recruitment and binding of 14-3-3 proteins (Cavadas et al., 2017; Grieve et al., 2017), which impact signalling by acting as adapters, masking binding sites, or preventing conformational changes (Pennington et al., 2018). In this case, binding of the 14-3-3 protein promotes stimulation of ADAM17 activity while also weakening the bond between ADAM17 and iRHOM2. Dissociation of this complex may be important to allow for ADAM17 binding to substrates to enable cleavage (Cavadas et al., 2017). At this point however, little is known about how the iRHOMs might interact with the ADAM17 substrates.

There is also some evidence to suggest that iRHOMs may also play a role in the substrate specificity and selectivity of ADAM17 at the cell membrane. For example, ADAM17 in complex with iRHOM1 has a slightly different substrate specificity to ADAM17 in complex with iRHOM2 (Maretzky et al., 2013). Site-directed mutagenesis studies implicate the first (Tang et al., 2020) and seventh (Zhao et al., 2022) transmembrane domain of the iRHOM in substrate selectivity and presentation to the activated ADAM17 catalytic site. Zhao *et al.* (2022) propose that the iRHOM2 (but not iRHOM1) positions iRHOM2-specific substrates so that their cleavage site is accessible to stimulated ADAM17. However, much more work is needed to further understand how the iRHOMs interact with ADAM17 substrates.

Moving on from the iRHOMs, there are a number of other interactions and conformational changes that can regulate ADAM17 activity on the cell surface.

The disintegrin domain of ADAM17 has been shown to interact with $\alpha_5\beta_1$ integrins in either cis (on the same cell as the ADAM17) or in trans (on a neighbouring cell) (Bax et al., 2004). This inhibits ADAM17 activity by limiting accessibility to the catalytic site by steric hindrance (Gooz et al., 2012).

The membrane-proximal domain along with the CANDIS is also involved in regulation of ADAM17 activity. These 2 regions form electrostatic interactions with the cell membrane that can change the conformation of ADAM17 and hence alter its activity. There is some evidence to suggest that ADAM17 is inactive when it is localised in cholesterol-rich

microdomains of the membrane, while localisation in a more phosphatidylserine-rich microdomain enhances its activity (Tellier et al., 2006, 2008). A short region in the membrane proximal domain is important for this localisation as it binds to phosphatidylserine on the outer layer of the membrane (Sommer et al., 2016). Deletion of the phosphatidylserine-binding motif from the membrane proximal domain results in embryonic lethality in mice (Veit et al., 2019), indicating it is crucial for ADAM17 activity. The membrane proximal domain can also be acted on by the enzyme protein disulfide isomerase, which catalyses the production of 2 disulfide bridges within this region and inhibits ADAM17 activity (Willems et al., 2010).

Post-translation down-regulation of ADAM17 activity can be achieved by interaction with the endogenous inhibitor TIMP-3 (Section 1.3), or by shedding of the ADAM17 ectodomain by ADAM8 (Scharfenberg et al., 2020). Removal of ADAM17 from the cell surface prevents it from targeting many of its substrates, since the soluble form of ADAM17 has a somewhat different substrate repertoire (Scharfenberg et al., 2020).

ADAM17 activity can also be regulated at the level of substrate structure. ADAM17 cleaves its substrates at a fixed distance close to the cell membrane and has no specific cleavage site motif (Lichtenthaler et al., 2018). This is one of the reasons why ADAM17 can cleave so many different substrates. However, some mechanisms have been proposed which could explain substrate susceptibility. It has been observed that ADAM17 cleavage sites are often located close to sites of O-glycosylation, and ablation of O-glycosylation reduced cleavage of several ADAM17 substrates, including TNF (Goth et al., 2015). ADAM17 has also been shown to have low activity on proteins that contain regions of negatively charged amino acids in their stalk regions. For example, activated leukocyte cell adhesion molecule, which is a substrate of ADAM17, has 2 splice variants, and the variant containing an exon that introduces a large number of negatively charged amino acids into the stalk region has reduced shedding (Iwagishi et al., 2020).

A plethora of mechanisms regulating ADAM17 activity have thus been described, and this is still an active area of research.

1.2.3 Differences and similarities between ADAM17 and ADAM10

ADAM17 and ADAM10 are the most well-studied and best characterised of the ADAMs. They are very closely related and based on molecular evolutionary analyses of the sequences of their metalloprotease domains, are a separate subgroup of the ADAMs (Edwards et al., 2009). As with ADAM17, ADAM10 has numerous substrates, with proteomic and mass spectrometry analysis identifying over 90 different substrates for

ADAM10, many of which have important roles in various signalling pathways and cellular processes (Kuhn et al., 2016).

ADAM10 and ADAM17 have many similar structural features and as a consequence they share many common substrates and often overlap in their shedding properties (Werny et al., 2022). For example, both ADAM17 and ADAM10 can cleave the IL-6R, albeit at different sites (Garbers et al., 2011; Riethmueller et al., 2016), and both can act as α-secretases of the amyloid precursor protein, producing the soluble form of the protein that promotes the non-amyloidogenic pathway and decreases formation of amyloid β (Asai et al., 2003; Werny et al., 2022). ADAM17 and ADAM10 can also both cleave CD137 (Schwarz et al., 1993), a member of the TNF receptor family expressed on many cell types in the immune system that stimulates cytokine secretion, expression of antiapoptotic molecules, cell proliferation, and effector function (Choi & Lee, 2020). Cleavage of CD137 by ADAM10 is stimulated by interaction of its membrane-proximal domain with phosphatidylserine residues on the cell membrane (Bleibaum et al., 2019; Seidel et al., 2021) as described above for stimulation of ADAM17 (Sommer et al., 2016).

Like ADAM17, ADAM10 can itself be shed by ADAM8, with the soluble protease retaining some catalytic activity, albeit with an altered substrate pool (Scharfenberg et al., 2020). The localisation of ADAM10 on the cell membrane and the membrane-associated proteins it interacts with can also affect its activity in a similar fashion to ADAM17 (Tosetti et al., 2021). However, many of the specific interactions on the cell membrane are unique to the ADAM.

There are some key differences in how ADAM17 and ADAM10 act. ADAM10 is generally considered to be a constitutive shedder while ADAM17 is much more sensitive to stimulation (Ludwig et al., 2005). ADAM17 activity can be stimulated in vitro using PMA as mentioned above (Section 1.2.2), while ADAM10 can be stimulated by cellular influx of Ca^{2+} in response to calcium ionophores like ionomycin (Horiuchi et al., 2007). The cytoplasmic domain of ADAM10 is necessary for the regulation of ADAM10-mediated constitutive shedding, while it is not involved in the rapid activation of ADAM10-mediated stimulated shedding (Maretzky et al., 2015).

While ADAM10 and ADAM17 share several substrates, there are a number of substrates that are unique to the individual ADAM. N-cadherin and betacellulin are specific to ADAM10, while TNF, amphiregulin, and heparin-binding EGF-like growth factor are specific to ADAM17 (Pruessmeyer & Ludwig, 2009).

The phenotype of *Adam10* knockout mice is even more severe than that of the *Adam17* knockout, with embryonic lethality caused by impaired development of the central nervous system and heart, replicating the phenotype of Notch deficiency and indicating that ADAM10 plays a central role in Notch signalling (Hartmann et al., 2002).

Various conditional or inducible *Adam10* knockout mice lines have been generated using Cre/loxP recombination methods in order to study the function of ADAM10 in specific cell types. Conditional inactivation of *Adam10* in the endothelial cells resulted in a phenotype which mirrored that of impaired canonical Notch signalling, demonstrating the key role of ADAM10 in regulating endothelial cell-fate decisions (Glomski et al., 2011). In a mouse model of Alzheimer's disease, the expression of a catalytically inactive form of ADAM10 resulted in an increased number and size of amyloid plaques showing ADAM10's role as an α-secretase (Postina et al., 2004).

ADAM10 and ADAM17 also differ in how their activities are regulated allowing for precise control of substrate shedding in response to a variety of different stimuli (Le Gall et al., 2009). For example, both ADAMs are able to shed the transmembrane enzyme carbonic anhydrase IX with overlapping cleavage sites (Zatovicova et al., 2023). This is a hypoxia-induced enzyme associated with cancer that plays a role in pH regulation, metabolic adaptation to hypoxia, and cell-migration/invasion (Pastorek & Pastorekova, 2015). The different regulatory mechanisms that control the activation of ADAM10 and ADAM17 therefore mean that carbonic anhydrase IX shedding can be controlled in a variety of tumour tissue contexts where there may be different levels of expression of ADAM10 or ADAM17, or of their activators or inhibitors.

In a similar fashion to how ADAM17 maturation, localisation, and activity is regulated by the iRHOMs, the interaction between ADAM10 and one of the C8 family of tetraspanins (TspanC8) is essential for regulating ADAM10 function (Matthews et al., 2017). Tetraspanins (Tspan) are membrane spanning proteins which primarily function as organisers of proteins within the plasma membrane. There have been 33 tetraspanins identified in humans some of which are ubiquitously expressed, while others are more tissue-specific (de Winde et al., 2015). As the name suggests, tetraspanins contain 4 transmembrane domains which creates two extracellular loops, one small and one large (Termini & Gillette, 2017), and cytoplasmic amino and carboxyl termini. They generally function through their interactions with other proteins thereby facilitating their intracellular trafficking and localisation on the cell surface. One example includes the tetraspanin CD151, which is involved in the assembly of multi-protein complexes called tetraspanin enriched domains which regulate laminin-binding integrins (Stipp, 2010). CD151 directly binds to the α3 subunit of α3β1 integrin and by linking to other tetraspanins and integrins affects cell motility (Gustafson-Wagner & Stipp, 2013). Loss of CD151 in a breast carcinoma model results in reduced migration, adhesion, EGF-induced spreading, and subcutaneous tumour growth (Baldwin et al., 2008).

Another example of tetraspanin function is CD81 which along with its binding partner CD19, form part of the B-cell co-receptor complex (Susa et al., 2021). CD19 belongs to the Ig

superfamily of proteins and is a single-pass membrane protein responsible for the threshold for B-cell receptor signalling. Stimulation of CD19 lowers the threshold needed for B-cell activation both by antigen dependent and independent mechanisms (Gauld et al., 2002). CD81 binding to CD19 is required for the trafficking of CD19 to the cell surface and so acts to chaperone CD19 through the secretory pathway. The interaction continues at the cell surface in a dynamic fashion which allows for the regulation of CD19 localisation in response to B-cell activation (Susa et al., 2020). In both of these cases, mutations of the tetraspanin involved results in a pathology which is consistent with an impairment of the partner protein (Karamatic Crew et al., 2004; Van Zelm et al., 2010). This demonstrates the importance of tetraspanins to their binding partner and their central role in many cellular processes.

The interaction between tetraspanins and ADAM10 involve a particular subgroup of the tetraspanins termed the C8 family (Matthews et al., 2017). The six tetraspanins in this group (5, 10, 14, 15, 17, 33) are unique from the other tetraspanins through the eight cysteine residues which are present in their large extracellular region. The TspanC8s are so important to ADAM10 function, that it has been proposed that ADAM10 exists as six distinct protease complexes depending on which regulatory TspanC8 is associated with it (Harrison et al., 2021; Matthews et al., 2018). Different cell types have different levels of expression of the different TspanC8s which may go some way to explaining which TspanC8 preferentially binds to ADAM10 in different contexts (Haining et al., 2012). The precise mechanisms to regulate which TspanC8 binds to ADAM10 are currently unknown.

The TspanC8s are responsible for regulating the maturation, trafficking, localisation, and substrate selectivity of ADAM10 (Figure 1.5) and so play a major role in regulating the cleavage of a number of substrates. The cleavage of N-cadherin by ADAM10 has been shown to be dependent on the Tspan15/ADAM10 complex and is not affected by the overexpression or knockdown of the other TspanC8s (Jouannet et al., 2016; Noy et al., 2016; Prox et al., 2012). The atomic model of the Tspan15/ADAM10 complex has recently been determined by cryo-electron microscopy showing two binding interfaces between the two proteins (Lipper et al., 2023). This structure demonstrates how the binding of Tspan15 to ADAM10 changes the conformation of ADAM10 from a closed to an open conformation, revealing the active site for membrane-proximal substrate cleavage. This provides a mechanism for how TspanC8 binding can alter ADAM10 activity by changing the location and distance of the active site above the cell membrane. The cleavage of Notch protein by ADAM10 has also been shown to be regulated by TspanC8s but in a more complex manner. Tspan5 and Tspan14 overexpression resulted in an increase in ADAM10-mediated Notch cleavage while overexpression of Tspan15 or Tspan33 had the opposite result. Knockdown of Tspan15 also induced Notch cleavage (Dornier et al., 2012; Jouannet et al., 2016). This

demonstrates how which TspanC8 ADAM10 is bound to directly affects its substrate selectivity and that by changing the expression of the tetraspanins, one can change the activity of ADAM10, suggesting that the tetraspanin which ADAM10 is bound to is at least in part dependent on the relative levels of tetraspanin expression. More recently, Tspan15 and Tspan33 have been shown to regulate the ADAM10-mediated shedding of the platelet-activating collagen receptor GPVI in a compensatory fashion (Koo et al., 2022). Tspan15 had the most important role in this, and further investigation showed that the extracellular region of Tspan15 was required for GPVI cleavage and had this effect by enabling ADAM10 to access the cleavage site at a particular distance above the cell membrane. This is further supported by the reported structure of the Tspan15/ADAM10 complex showing an altered active site height above the membrane (Lipper et al., 2023).

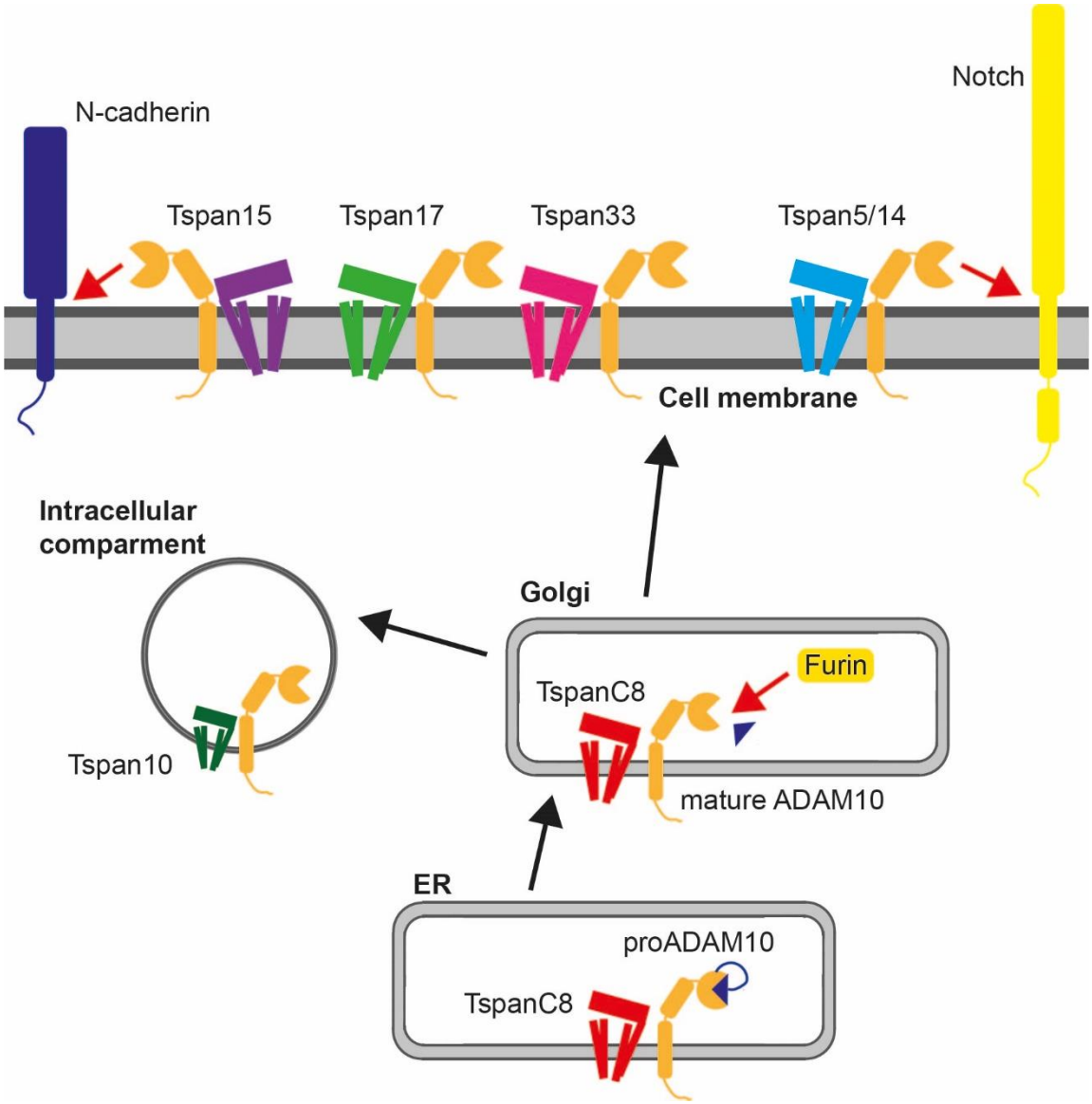

Figure 1.5 Regulation of ADAM10 by the TspanC8 family of tetraspanins

The interaction between ADAM10 and a TspanC8 is required for the trafficking and maturation of ADAM10 from the ER to the cell membrane. TspanC8s promote the trafficking

of ADAM10 from the ER and the removal of its pro-domain, and the majority of them promote trafficking to the cell membrane. The exception is Tspan10 which remains intracellular. The TspanC8 can then regulate the substrate specificity of the ADAM10 to which it is associated with.

1.3 TIMP-3 is the physiological inhibitor of ADAM17

One of the key mechanisms by which ADAMs and MMPs are physiologically regulated is by interaction with their endogenous inhibitors, the TIMPs. There are four mammalian TIMPs, but only TIMP-3 appreciably inhibits ADAM17 (Amour et al., 1998). TIMP-3 has a broad inhibitory profile and is able to target several members of the ADAM, MMP, and ADAMTS families (Brew & Nagase, 2010). As a consequence of this, TIMP-3 plays an important role in extracellular matrix (ECM) turnover and tissue remodelling in addition to its regulation of ADAM17 activity. TIMP-3 is also the only member of the TIMPs that can bind to the ECM (Yu et al., 2000).

TIMP-3 forms strong non-covalent bonds with a 1:1 stoichiometry with the active site of ADAM17, inhibiting its proteolytic activity (Amour et al., 1998). The functional binding edge of TIMP-3, which consists of an N-terminal region and other loops, interacts directly with the active site of ADAM17 (Wisniewska et al., 2008).

The importance of TIMP-3 regulation of ADAM17 activity has been shown in various disease contexts using *Timp3* knockout mice. The first *Timp3* knockout mice created showed phenotypes consistent with enhanced matrix degradation due to a lack of metalloprotease inhibition (Fata et al., 2000; Leco et al., 2001). Deletion of *Timp3* in mice has also been shown to increase constitutive release of TNF as a result of reduced inhibition of ADAM17. A consequence of this increased TNF is chronic inflammation of the liver of *Timp3*-null mice, with lymphocyte infiltration and necrosis (Mohammed et al., 2004). This phenotype could be rescued by anti-TNF antibodies or by additional mutation of the TNFR, demonstrating the key role that TNF plays in tissue homeostasis and the importance of TIMP-3 in regulating its release. *Timp3*-null mice also exhibit prolonged inflammation after acute lung injury which could be rescued by treatment with an exogenous inhibitor of ADAM17 (Gill et al., 2010), showing that TIMP-3 helps resolve the inflammatory response through its ability to inhibit ADAM17. Furthermore, *Timp3*-null mice show increased diabetes (Federici et al., 2005), kidney fibrosis (Kassiri et al., 2009), atherosclerosis (Stöhr et al., 2014), susceptibility to LPS-induced mortality (Smookler et al., 2006), spontaneous generation of arthritis in aged mice (Sahebjam et al., 2007), and accelerated cardiac remodelling after myocardial infarctions (Tian et al., 2007).

Since TIMP-3 is the physiological inhibitor of ADAM17 (Black, 2004; Mohammed et al., 2004), it is crucial that levels of TIMP-3 are tightly controlled.

There are a number of methods by which the expression of *TIMP3* can be affected. For example, transcription of *TIMP3* is upregulated by the histone deacetylase Sirtuin 1 (Cardellini et al., 2009), and downregulated by promoter methylation (Darnton et al., 2005). Growth factors such as TGF-β and oncostatin M have been shown to promote *TIMP3* transcription (Li & Zafarullah, 1998; Su et al., 1998) and various micro-RNAs have been demonstrated to decrease translation (Gabriely et al., 2008; Wang et al., 2010). However, it was observed that increased TIMP-3 mRNA levels do not necessarily correlate with increased TIMP-3 protein levels (Troeberg et al., 2008), and it was subsequently established that TIMP-3 is primarily regulated post-translationally, by endocytosis and lysosomal degradation via the endocytic scavenger receptor low-density lipoprotein receptor related protein 1 (LRP1) (Troeberg et al., 2008; Scilabra et al., 2013; Doherty et al., 2016; Schubert et al., 2019). Scilabra *et al.* (2013) used radiolabelled recombinant TIMP-3 to demonstrate that TIMP-3 can be endocytosed by a variety of cell types, and that this process was blocked by the LRP1 antagonist receptor-associated protein (RAP). They also showed that TIMP-3 endocytosis was greatly reduced in LRP1-deficient PEA-13 mouse embryonic fibroblast (MEF) cells compared to wild-type MEF cells, identifying LRP1 as the primary mechanism of endocytosis. LRP1-independent endocytosis of TIMP-3 was observed, but this was considerably slower than the LRP1 mediated process. The rate of LPR1 mediated TIMP-3 endocytosis was not constant, and after an initial rapid phase, reduced to minimal levels of endocytosis by 10 h. Scilabra *et al.* (2013) showed that this reduced rate of endocytosis was due to shedding of LRP1, with LRP1 in the medium acting as a decoy receptor and increasing the half-life of TIMP-3 in the medium. Subsequent site-directed mutagenesis studies indicated that TIMP-3 binding to LRP1 is dependent on pairs of lysine residues located within the area of basic residues opposite the N-terminal inhibitory edge of TIMP-3 (Doherty et al., 2016).

TIMP-3 is also able to bind strongly to heparan sulfate proteoglycans in the ECM (Yu et al., 2000) and on the cell surface (Koers-Wunrau et al., 2013). TIMP-3 binds to heparin sulfate proteoglycans (HSPGs) via an area of basic residues removed from its inhibitory edge (Lee et al., 2007), and so retains its inhibitory activity when bound to HSPGs (Troeberg et al., 2014). The localisation of TIMP-3 on the cell surface in unclear – it is likely to bind to cell surface HSPGs such as syndecans, but it has also been shown that under resting, unstimulated conditions TIMP-3 can associate with dimerised ADAM17 in an inactive complex on the cell surface (Xu et al., 2014). Xu *et al.* (2014) proposed that when ADAM17 is stimulated, the complex dissociates, TIMP-3 is released, and ADAM17 monomers exhibit increased proteolytic activity.

Site-directed mutagenesis studies indicate that TIMP-3 binds to both HSPGs (Lee et al., 2007) and LRP1 (Doherty et al., 2016) via an area of basic residues opposite the N-terminal inhibitory edge of TIMP-3, and heparin and HSPGs have been shown to inhibit TIMP-3 binding to LRP1 (Troeberg et al., 2008; Scilabra et al., 2013; Doherty et al., 2016). TIMP-3 is thus likely to present on the cell surface in an equilibrium between target metalloprotease such as ADAM17, HSPGs, and LRP1.

1.4 The endocytic receptor LRP1

LRP1 is an endocytic scavenger receptor of the low density lipoprotein receptor family. It is widely expressed in tissues including the liver, brain, and lungs, and crucially for this project, is highly expressed in macrophages (Nilsson et al., 2012; Overton et al., 2007). As mentioned above, LRP1 plays a key role in regulating extracellular levels of the endogenous ADAM17 inhibitor TIMP-3, and so is important for helping to control the inflammatory response in macrophages (Schubert et al., 2019).

Over 90 LRP1 ligands have been identified, meaning that LRP1 plays a role in a wide range of cellular functions (Table 1.2) (Bres & Faissner, 2019). It also mediates the cellular entry of some viruses and toxins (Liu et al., 2000), and contributes to maintenance of the blood-brain barrier by transcytosing ligands across endothelial cells of the central nervous system (Strickland et al., 2014).

Table 1.2 Examples of LRP1 ligands.
LRP1 can interact with a variety of ligands at the cell surface and so influence many cellular functions (Bres & Faissner, 2019; Lillis et al., 2009; Yamamoto et al., 2022).

α1-antitrypsin	Heat shock protein 90, 96, 70	Ricin A
α1-antitrypsin:trypsin complexes α-2 macroglobulin	Heparan sulfate proteoglycans	Saporin
ADAMTS1, 5	Hepatic lipase	Saposin precursor
Amidoglycosides: gentamicin, polymixcin B	Hepatocyte growth factor activator	Secreted protein acidic and rich in cysteine
Amyloid β peptide	High mobility group box 1	Shc
Amyloid precursor protein	High mobility group box 2	Slit2
Annexin VI	HIV-Tat protein	Triglyceride-rich lipoproteins
Apolipoprotein E	Insulin	Tissue inhibitors of matrix metalloprotease 1, 2, 3

(ApoE)/ApoE-containing lipoproteins	Insulin-like growth factor-binding protein 3, 7	Tissue factor pathway inhibitor
Aprotinin	Lactoferrin	TpeL
Bone morphogenic factor 4	Leptin lipoprotein lipase	Transforming growth factor-β 1
BMP-binding endothelial cell precursor-derived regulator	Leukemia inhibitory factor	Transforming growth factor-β 2
C1s/C1q	Malaria circumsporozoite protein	Thrombospondin 1
C4b-binding protein	Matrix metalloproteinase 2	Thrombospondin 2
Calreticulin	Matrix metalloproteinase 9	Tissue-type plasminogen activator
Cathepsin D	Matrix metalloproteinase 13	tPA:PAI-1 complexes
Cysteine-rich angiogenic inducer 61	Metallothionein II	tPA:neuroserpin complexes
Cell migration-inducing and hyaluronan-binding protein	Midkine	Thrombin:protein inhibitor C complexes
Chylomicron remnants	Minor-group human rhinovirus	Thrombin:nexin-1 complexes
Coagulation factor VII	Myelin-associated glycoprotein	Thrombin:antithrombin III complexes
Coagulation factor Xa: tissue factor pathway inhibitor complexes	Myelin basic protein	Thrombin:heparin cofactor II complexes
Coagulation factor XIa:nexin complexes	Nexin-1	Thrombin:PAI-1 complexes
Complement component 3	Plasminogen activator inhibitor	Trichosanthin
Connective tissue growth factor	Platelet-derived growth factor-BB PDGF receptor-β	TNF-stimulated gene 6 protein
Decorin	Postsynaptic density protein 95	Urokinase-type plasminogen activator
Disabled 1	Prion protein	uPA:PAI-1 complexes
Fe-65	Pregnancy zone protein:protease complexes	uPA:PAI-2 complexes
Fibronectin	Pro-urokinase	uPA:C inhibitor complexes
Frizzled-1	Pseudomonas exotoxin A	uPA:Nexin-1 complexes
Glypican-3:Hedgehog complexes	Receptor-associated protein	Von Willebrand factor

Among the many ligands that can undergo LRP1-mediated endocytosis are numerous proteins involved in ECM degradation such as matrix-degrading aggrecanases (Yamamoto

et al., 2014) and collagenases (Carreca et al., 2020), so LRP1 plays a role in turnover and remodelling of the ECM. As I have discussed (Section 1.3), LRP1 can bind and endocytose TIMP-3 (Scilabra et al., 2013), and also endocytoses TIMP-1 and TIMP-2 (Table 1.2), and so impacts inhibition of these proteases and so further regulates ECM turnover. LRP1 can also endocytose proteins complexed with their membrane-associated receptors which further impacts on the protein composition of the ECM (Gonias et al., 2004). For example, urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) bind with greater affinity to LRP1 when also in complex with the receptor urokinase-type plasminogen activator receptor (uPAR) that results in LRP1-mediated endocytosis only taking place when PAI-1 binds to the uPA/uPAR complex (Czekay et al., 2001). LRP1 can therefore regulate uPAR-mediated proteolysis of the ECM. LRP1 control of ECM turnover has been shown to play an important role in a number of settings including cartilage and the central nervous system. An increase in the levels of the aggrecanase ADAMTS5 and the matrix metalloprotease MMP-13 in the ECM was found to be the result of reduced LRP1-mediated endocytosis and this resulted in increased ECM degradation leading to the progression of osteoarthritis (Yamamoto et al., 2017).

LRP1 is initially synthesised as a 600 kDa protein before furin-mediated proteolytic cleavage in the Golgi apparatus results in an N-terminal 515 kDa α-chain (heavy chain), and a C-terminal 85 kDa β-chain (light chain) (Figure 1.6). These 2 subunits are non-covalently associated to form the mature transmembrane LRP1 protein (Herz & Strickland, 2001; Lillis et al., 2008). The extracellular heavy chain is composed of 4 clusters (named I-IV) of ligand-binding repeats separated by EGF homology domains. These clusters contain 2, 8, 10, and 11 ligand-binding repeats, which are the binding sites for the many ligands of LRP1. While the clusters are fairly similar in terms of their structures, there are differences in their affinity for individual ligands due to the variation in repeats per cluster (Neels et al., 1999). These differences result in each ligand-binding repeat having different charge densities and hydrophobic patches that alter ligand binding (Huang et al., 1999). Clusters II and IV are generally considered to bind the majority of LRP1 ligands (Croy et al., 2003; Meijer et al., 2007) and TIMP-3 has been shown to preferentially bind to cluster II (Scilabra et al., 2017). The chaperone protein RAP, which assists in LRP1 folding and trafficking through the secretory pathway, is able to bind strongly to all the ligand-binding domains and completely block the binding of any other ligands (Bu et al., 1994; Williams et al., 1992) making it a useful experimental tool for antagonising LRP1-mediated ligand endocytosis in vitro.

The light chain of LRP1 spans the plasma membrane and extends intracellularly. It contains 2 NPxY motifs, 1 YxxL motif, and 2 di-leucine motifs. These motifs have been demonstrated to be involved in the rapid endocytosis of LRP1 (Deane et al., 2008) and the C-terminal can

also bind intracellular ligands, scaffold proteins, and other membrane proteins (Betts et al., 2008; Guttman et al., 2009).

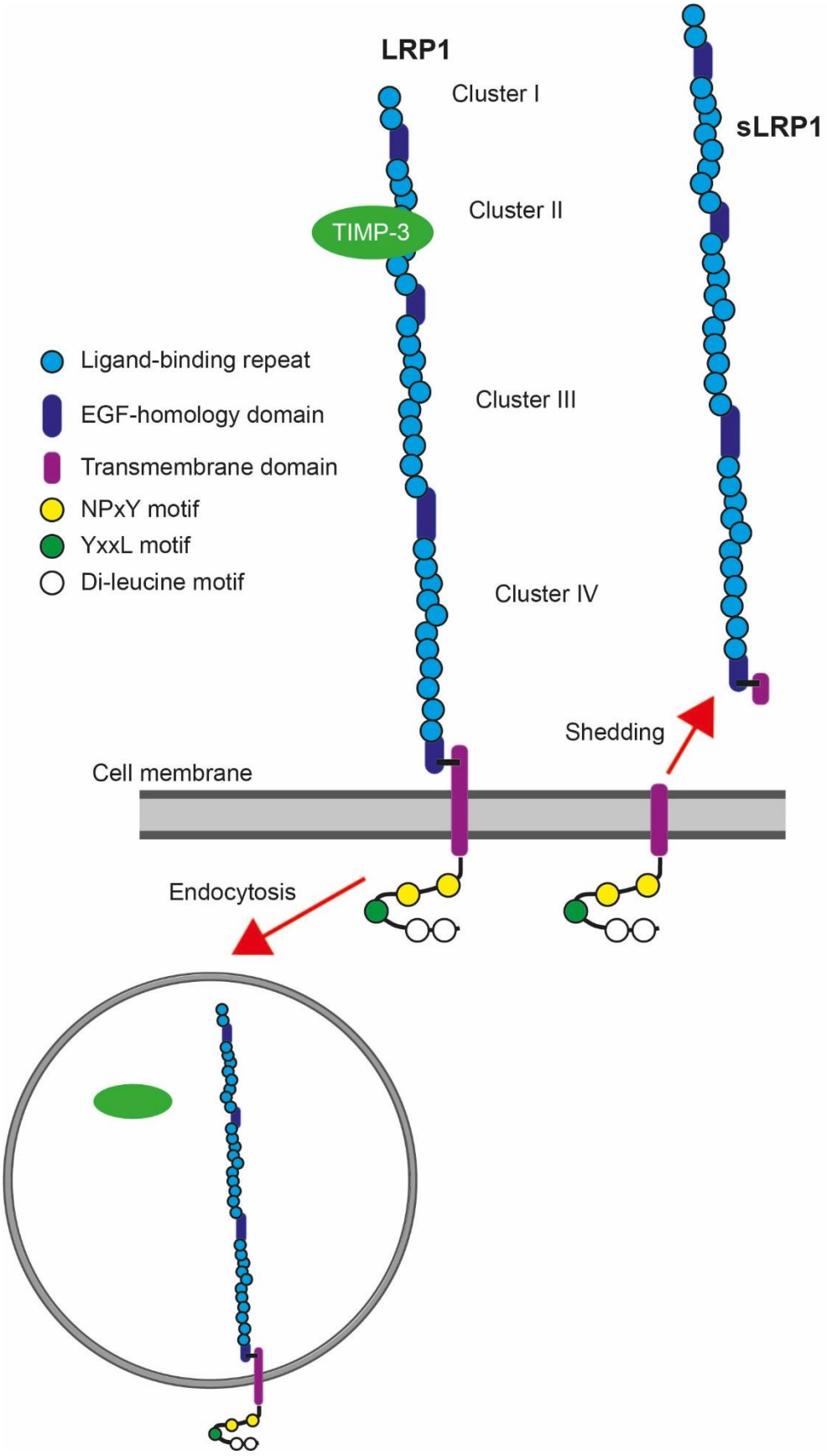

Figure 1.6 LRP1 can either endocytose its ligands or be cleaved and shed from the cell surface.

The α-chain of LRP1 contains 4 clusters of ligand-binding repeats to which ligands (e.g. TIMP-3) can bind allowing for their LRP1-mediated endocytosis and subsequent lysosomal

degradation. LRP1 β-chain can be proteolytically cleaved at the cell membrane, shedding the soluble form of LRP1 from the cell. Shed sLRP1 retains its ligands-binding properties and so can still interact with ligands in the ECM and circulation.

1.4.1 Roles of LRP1 in inflammation

Increased expression of LRP1 has been observed in a number of chronic inflammatory conditions, such as atherosclerosis (Llorente-Cortés et al., 2004; Luoma et al., 1994) and multiple sclerosis (Hendrickx et al., 2013), and increased shedding of LRP1 has also been observed in multiple chronic inflammatory conditions, including RA, systemic lupus erythematous (Gorovoy et al., 2010), liver disease (Quinn et al., 1997), and acute respiratory distress syndrome (Wygrecka et al., 2011).

Studies on several murine models and disease contexts have shown that LRP1 has a complex role in inflammation. Complete knockout of *Lrp1* in mice is lethal to the embryo (Herz et al., 1992, 1993), so mice with conditional and cell specific *Lrp1* deletion have been generated to study its role. *LRP1* is widely expressed but is most abundant in adipocytes and CD14+ myeloid cells (Su et al., 2004), so several groups have focused on the role of LRP1 in macrophages and in atherosclerosis in particular. Most of these studies suggest an anti-inflammatory role for LRP1 in macrophages, but there is also evidence for a pro-inflammatory role in some contexts.

LRP1-deficient macrophages release more TNF in response to LPS stimulation in vitro (Overton et al., 2007; Zurhove et al., 2008) and atherosclerosis is increased in ApoE-null mice with macrophage *Lrp1*-deficiency (Hu et al., 2006). Accelerated atherosclerotic plaque growth was associated with increased TNF release, along with macrophage apoptosis as a result of protein kinase B inhibition, and decreased efferocytosis (Overton et al., 2007; Yancey et al., 2010). Similarly, May *et al*. (2013) showed that LRP1-deficient murine macrophages express lower levels of anti-inflammatory markers and have enhanced response to pro-inflammatory stimuli (May et al., 2013). Primary human monocytes isolated from patients with sub-clinical atherosclerosis express less LRP1 at the mRNA and protein level compared with healthy controls, and this reduction was associated with increased expression of both *TNF* and *IL1β* (Albertini et al., 2022).

Various molecular mechanisms for this anti-inflammatory effect have been proposed, including suppression of TNFR1-dependent NF-κB activity through the reduction of cell surface TNFR1 levels by an unknown mechanism (Gaultier et al., 2008) and transcriptional suppression of the interferon gamma promoter by the LRP1 intracellular domain (Zurhove et al., 2008)(Section 1.4.3).

More recently, LRP1 was proposed to inhibit TNF release via its ability to control cell surface levels of TIMP-3, hence regulating the activity of ADAM17 in macrophages (Schubert et al., 2019)(Figure 1.7). This model suggests that shedding of LRP1 in response to LPS leads to accumulation of TIMP-3 on the macrophage cell surface, inhibiting ADAM17 and so halting TNF release.

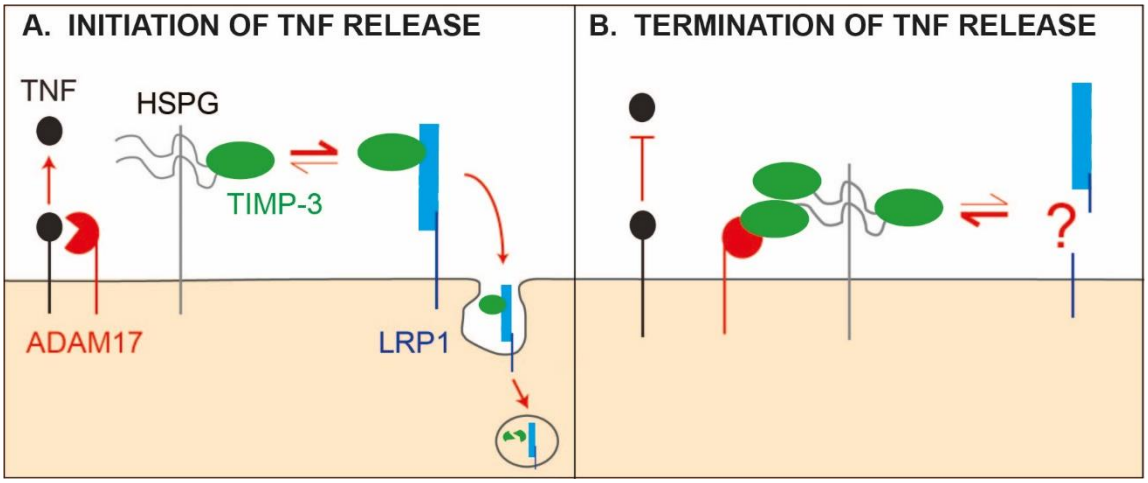

Figure 1.7 Model proposed by Schubert *et al.* (2019) for LRP1 regulation of TNF release via TIMP-3/ADAM17.

Schubert *et al.* (2019) found that LRP1 controls cell surface levels of TIMP-3 in LPS-stimulated macrophages, and so regulates ADAM17-mediated release of TNF. They propose that shedding of LRP1 results in an accumulation of TIMP-3 on the cell surface, inhibiting ADAM17-mediated TNF release, and contributing to the resolution of the inflammatory response. However, the protease responsible for shedding LRP1 from human macrophages is currently unknown.

However, other studies have indicated a pro-inflammatory role for LRP1 in macrophages. In vitro studies indicate that addition of LRP1 ectodomain to macrophages stimulated pro-inflammatory signalling through p38 MAP kinase and c-Jun N-terminal kinase (JNK) (Gorovoy et al., 2010). It is not clear what receptors the shed LRP1 signals through, but an antibody targeting the LRP1 N-terminus was able to inhibit this signalling. LRP1 shedding is increased by exposure to pro-inflammatory mediators like LPS and IFN-γ, suggesting that LRP1 can participate in a feed-forward mechanism that amplifies inflammation. This is supported by the observation that regression of atherosclerotic plaques is accelerated in mice with LRP1-deficient macrophages, with increased egress of macrophages from plaques (Mueller et al., 2018).

1.4.2 Regulation of LRP1-mediated ligand endocytosis

LRP1 can internalise its bound ligands by endocytosis via clathrin-coated pits (Lillis et al., 2005). This process requires the NPxY motifs in the cytoplasmic region of the β-chain, and when these domains are mutated, internalisation of ligands is greatly reduced (Chen et al., 1990). How the 2 NPxY motifs regulate endocytosis is not fully understood, however they have been shown to interact with multiple intracellular signalling proteins through tyrosine phosphorylation at Tyr 4507 and subsequently, Tyr 4473 (Betts et al., 2008; Guttman et al., 2009). Knock-in mutation of the NPxY motifs have been shown to impair LRP1-mediated endocytosis and interfere with downstream signalling pathways (Roebroek et al., 2006). Serine and threonine phosphorylation by protein kinase Cα may also be involved in switching from endocytosis to acting as a signalling receptor (Ranganathan et al., 2004). Endocytosed ligands are delivered to endosomes, and then degraded by lysosomes or the proteosome, while LRP1 is recycled back to the cell membrane (Bres & Faissner, 2019).

Alternatively, LRP1 can itself be degraded by the proteasomal system instead of being recycled, providing a mechanism to regulate cell surface levels of LRP1. In the J774 macrophage-like cell line, it was demonstrated that insulin treatment induced degradation of LRP1 by the proteosome, and consequently reduced internalisation of LRP1 ligands (Ceschin et al., 2009). Conversely, in other cell types, the opposite effect has been observed. For example, in adipocytes, insulin was found to stimulate recycling of LRP1 from the endosome back to the cell membrane (Ko et al., 2001). These differences highlight the important and complex role LRP1 plays in regulating many cellular functions, often in a cell-specific manner.

As well as its soluble ligands, LRP1 can also interact with ECM components. LRP1-mediated endocytosis of some ligands is only possible when they are first weakly bound to HSPGs (Kanekiyo et al., 2011). Endocytosis is blocked if this binding is inhibited by treatment with heparin. LRP1 has also been shown to associate directly with HSPGs and by doing so enhance endocytosis of some of its ligands (Spijkers et al., 2008; Taylor & Hooper, 2007; Wilsie & Orlando, 2003). As mentioned above (Section 1.3), endocytosis of other ligands, such as TIMP-3, is inhibited by ligand binding to heparin or HSPGs. This apparent discrepancy reflects variation in whether LRP1 and HSPG binding sites on a ligand overlap or a structurally distinct from each other.

Another mechanism by which LRP1-mediated endocytosis of ligands is regulated is by localisation of LRP1 on the cell membrane. The distribution of LRP1 on the cell membrane is highly dynamic and has been shown to be cell type-specific (L. Wu & Gonias, 2005). Most studies indicate that in order for LRP1 to be endocytosed, it needs to translocate to clathrin-coated pits within the membrane (Bres & Faissner, 2019) and in vascular smooth muscle

cells, LRP1 was almost solely localised to these coated pits (Weaver et al., 1996). In both human fibroblasts and neurons, LRP1 was shown to be localised not only to coated pits, but also to areas of the membrane which were high in cholesterol and sphingolipids (Boucher et al., 2002; Laudati et al., 2016). These lipid rafts were found to be necessary for LRP1-mediated signalling but were not involved in ligand binding or endocytosis. However, LRP1 endocytosis has also been observed in lipid rafts via a caveolin-dependent pathway in hepatic cells and mouse embryonic fibroblasts (Kanai et al., 2014; Zhang et al., 2004). LRP1 has also been observed to concentrate at sites of cilia assembly in murine and human chondrocytes, and mutation of the cilia protein intraflagellar transport 88 (IFT88) was found to disrupt this localisation and impair LRP1-mediated endocytosis (Coveney et al., 2018). Laudati *et al.* (2016) proposed that the different functions of LRP1 at the cell membrane require different membrane localisations, and this again appears to vary with cell type.

1.4.3 Regulation of LRP1 function by shedding

The function of LRP1 can also be regulated by proteolytic shedding of its ectodomain, which releases a soluble form of the receptor, sLRP1 (Grimsley et al., 1998; Quinn et al., 1997, 1999).

LRP1 is most commonly cleaved at a single membrane-proximal site, releasing sLRP1 composed of the 515 kDa α-chain, and the 55 kDa extracellular portion of the β-chain (Quinn et al., 1997, 1999). Several studies have reported extracellular breakdown of sLRP1 into smaller fragments (Liu et al., 2009; Scilabra et al., 2013). Small sLRP1 fragments can also be generated directly from transmembrane full-length LRP1 by membrane-type MMPs (Rozanov et al., 2004). Many of the larger fragments of sLRP1 retain ligand-binding ability, and so can act as competitive inhibitors for ligand endocytosis (Selvais et al., 2009; Wygrecka et al., 2011). This means that LRP1 shedding reduces endocytosis of ligands in 2 complementary ways, i.e. by reducing the endocytic capacity of the cell and by generating a decoy receptor.

A further consequence of LRP1 shedding is that it enables subsequent γ-secretase-mediated release of the LRP1 intracellular domain (May et al., 2002). γ-secretase is an intramembranous protease that cleaves single-pass transmembrane proteins in their transmembrane portion, releasing an intracellular fragment that translocates to the nucleus to regulate transcription (Ehebauer et al., 2006). It was first identified as playing a role in Notch signalling (Ehebauer et al., 2006), and is now known to act similarly on multiple transmembrane proteins, including LRP1.

Upon cleavage of the LRP1 β-chain by γ-secretase, the intracellular domain of LRP1 translocates to the nucleus, where it can bind to transcription factors, facilitating their

nuclear export (Zurhove et al., 2008). For example, the LRP1 intracellular domain can bind to the transcription factor IRF3, which is a key transcription factor driving expression of genes in response to type 1 IFN. This interaction inhibits the binding of IRF-3 to its transcriptional coactivators CBP/p300, and subsequently facilitates the nuclear export of IRF-3, reducing the expression of IRF-3 target genes. The LRP1 intracellular domain can also bind directly to the IFN-γ promotor, limiting its transcription. As γ-secretase-mediated cleavage of the intracellular domain is dependent on prior shedding of the extracellular domain of LRP1 which is stimulated by pro-inflammatory factors, this mechanism acts as a negative feedback loop to limit inflammation.

Similarly, the intracellular domain of LRP1 can bind to the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ), which is a central regulator of lipid and glucose metabolism (Mao et al., 2017). LRP1 shedding can thus regulate cell signalling not only by reducing endocytosis of ligands and producing a decoy receptor, but also by enabling generation of the intracellular domain, which can suppress the expression of target genes.

LRP1 shedding can be either constitutive or rapidly stimulated by LPS or IFN-γ (Gorovoy et al., 2010; LaMarre et al., 1993), in line with the altered shedding in inflammatory conditions that I have previously discussed (Section 1.4.1). The rate at which LRP1 is shed is dependent on its glycosylation status, with glycosylation decreasing proteolytic cleavage (May et al., 2003). LRP1 is differentially glycosylated in different tissues, so this could contribute to observed differences in the rate of LRP1 shedding in different cell types.

A number of different proteases have been proposed to shed LRP1, with different sheddases identified in different cell types. The reasons for this variability have not yet been systematically investigated, but it is likely that numerous proteases are capable of cleaving LRP1, and that regulation of LRP1 shedding is cell specific.

The transmembrane protease β site of APP-cleaving enzyme (BACE) was the earliest proposed LRP1 sheddase (von Arnim et al., 2005). BACE is an aspartyl protease that cleaves the amyloid precursor protein with β-secretase activity. Von Arnim *et al.* (2005) observed that BACE interacts with the LRP1 β-chain in lipid rafts of H4 neuroglioma cells. They demonstrated that this interaction increased levels of LRP1 shedding. However, shedding was measured using an engineered-reporter, consisting of alkaline phosphatase fused to the N-terminus of the LRP1 β-chain. This is an indirect method of measuring LRP1 shedding, and the study did not include direct measurement of shed LRP1 α-chain in the conditioned media of cells, so additional investigation is required to clarify the role of BACE as an LRP1 sheddase.

The serine protease tissue plasminogen activator (tPA) has also been implicated in the shedding of LRP1, both in primary murine astrocytes and in an astrocyte cell line

(Polavarapu et al., 2007). This protease, which belongs to the chymotrypsin family, is primarily known for its role in degrading ECM components via activating plasmin, which in turn activates several MMPs (Kagitani et al., 1985). Polavarapu *et al.* (2007) observed reduced LRP1 shedding in *tpa* knockout mice, and concluded that tPA sheds LRP1. However, tPA is also an LRP1 ligand (Orth et al., 1992), and some of the other LRP1 ligands [e.g., RAP and calreticulin (Brifault et al., 2017)] have been observed to induce LRP1 shedding. Further studies are thus also warranted to determine whether tPA acts as a direct sheddase of LRP1, or whether it indirectly induces shedding by other protease(s) as a result of binding as a ligand and potentially inducing conformational changes that favour shedding.

Conversely, a number of studies have clearly shown that metalloproteases can act as LRP1 sheddases. Liu *et al.* (2009) demonstrated a role for both ADAM10 and ADAM17 in shedding LRP1 in a MEF cell line. In MEF cells lacking either ADAM10 or ADAM17, they observed a reduction in the extracellular levels of sLRP1 relative to the cellular levels. Overexpression of ADAM10 or ADAM17 also increased the levels of sLRP1, and this effect was inhibited by the co-overexpression of TIMP-3.

The role of metalloprotease(s) in shedding LRP1 was further demonstrated in the murine macrophage cell line RAW 264.7. Addition of metalloprotease inhibitor GM6001 inhibited both LPS- and IFN-γ-stimulated shedding of LRP1 (Gorovoy et al., 2010). Using small interfering RNA (siRNA) knockdown of A*dam9, Adam10,* and A*dam17,* Gorovoy *et al.* (2010) showed that ADAM17 alone was responsible for this stimulated shedding, while the constitutive shedding was unaffected by any of the conditions tested.

GM6001 was also shown to inhibit LRP1 shedding from HT1080 human fibrosarcoma cells. This was further investigated using other metalloprotease inhibitors, and both the broad-spectrum metalloprotease inhibitor BB-94 and the ADAM inhibitor KB-R7785 were found to be effective (Selvais et al., 2011). Unlike in the murine macrophages however, more specific targeting methods such as blocking antibodies and siRNA showed that ADAM12 and membrane type 1 matrix metalloprotease (MT1-MMP) were responsible for LRP1 shedding in HT1080 cells. This shedding was stimulated by cholesterol depletion, and not altered by inflammatory cytokines. Use of siRNA and blocking antibodies indicated MT1-MMP and ADAM17 are responsible for LRP1 shedding in chondrocytes (Yamamoto et al., 2017). The variation in LRP1 sheddases identified could indicate that different proteases are responsible for constitutive shedding and induced shedding, and that these additionally vary with cell type. This complex regulatory mechanism is likely to reflect the central role LRP1 plays in diverse cellular processes, including ECM turnover and signalling.

1.5 Hypothesis and aims of project

LRP1 has been shown to regulate levels of TIMP-3 in macrophages, and hence to regulate TNF release by the TIMP-3 target enzyme, ADAM17. Furthermore, LRP1 activity in multiple cell types is known to be modulated by shedding of the receptor.

In other cell types and species, a number of different proteases have been identified as LRP1 sheddases, with metalloproteases most commonly implicated. In murine macrophages, ADAM17 is responsible for LRP1 shedding, but the enzyme responsible for shedding LRP1 in human macrophages is still unknown.

I therefore hypothesised that a metalloprotease was most likely to shed LRP1 in human macrophages, and considered ADAM17 to be the most likely candidate. This would mean that ADAM17 activity is in part regulated by a negative feedback mechanism through LRP1/TIMP-3.

Therefore, my aims were to:

1. Investigate whether LRP1 shedding from human macrophages is stimulated in response to pro-inflammatory stimuli.
2. Test which protease or proteases are responsible for shedding LRP1 from human macrophages.

Chapter 2: Materials and Methods

2.1 Cell line culture

THP-1 human monocytic cells [from laboratory stocks purchased from the American Type Culture Collection (ATCC)] were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Thermofisher Scientific 11530586) supplemented with 10% fetal bovine serum [FBS (Merck F9665)], 1% penicillin-streptomycin [PenStrep (Lonza LZDE17-602E)], non-essential amino acid [NEAA (Gibco 11350912)], 1 mM sodium pyruvate (Gibco 12539059), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES (Gibco 11560496] in a humidified atmosphere at 37 °C with 5% CO_2. U937 human monocytic cells (from laboratory stocks purchased from ATCC) were cultured in RPMI 1640 medium, 10% FBS, 1% PenStrep, in a humidified atmosphere at 37 °C with 5% CO_2. RAW 264.7 murine monocyte/macrophage cells (gift from Anastasia Sobolewski, University of East Anglia) were cultured in RPMI 1640 medium, 10% FBS, 1% PenStrep, in a humidified atmosphere at 37 °C with 5% CO_2. L929 murine fibroblast cells (gift from Jelena Gavrilovic, University of East Anglia) were cultured in high glucose Dulbecco's Modified Eagle Medium [DMEM (Scientific Laboratory Supplies LZBE12-604F)], 10% FBS, 1% PenStrep, in a humidified atmosphere at 37 °C with 5% CO_2. All cell lines were cultured in 10 cm cell culture dishes (Greiner Bio-One 664160) and passaged once 80% confluency was reached. Trypsin-Ethylenediaminetetraacetic acid (EDTA) 0.25% (Gibco 2520056) was used to dissociate adherent cells for passaging.

2.2 Isolation of primary human macrophages

Leukocyte cones were obtained from National Health Service (NHS) Blood and Transplant (NC24) under Faculty of Medicine and Health Sciences Research Ethics Committee approval 2019/20-097 and NHS Blood and Transplant approval R347. Blood was drained from the cones and made up to 40 mL with phosphate buffered saline (PBS) lacking calcium and magnesium (Gibco 15326239). Blood was layered on top of 2 x 20 mL Ficoll-Paque PLUS (Cytiva 11768538) and centrifuged [400 relative centrifugal force (rcf), 35 min, 21 °C, acceleration 5, deceleration 0] before the interface layer of peripheral blood mononuclear cells was isolated and made up to 40 mL with Hank's balanced salt solution (HBSS) lacking calcium and magnesium (Gibco 11590466). Cells were centrifuged (80 rcf, 10 min, 21 °C), supernatants discarded, and pellets resuspended in HBSS followed by a further centrifugation step (80 rcf, 10 min, 21 °C) to wash the cells. Pellets were resuspended in serum-free RPMI 1640 media and diluted to $20x10^6$ cells/mL before being layered on top of 2 x 20 mL Percoll solution [45% Percoll PLUS (Cytiva 11500744), 10% 10x PBS]. Cells were centrifuged (400 rcf, 30 min, 21 °C, acceleration 0, deceleration 0) and the interface layer was isolated and made up to 50 mL with serum-free RPMI 1640 media. Cells were

centrifuged (80 rcf, 10 min, 21 °C) and resuspended in serum free RPMI 1640 media to a density of $2x10^6$/mL before being plated in 10 cm tissue culture plates and incubated (37 °C, 5% CO_2, humidified atmosphere) for 60 min to allow highly adherent cells to attach to the plate. Medium containing any non-adherent cells (mainly lymphocytes and neutrophils) was removed and fresh RPMI 1640 5% FBS was added, supplemented with either recombinant human macrophage colony-stimulating factor (M-CSF) (100 ng/mL, Peprotech 300-25) or 30% conditioned media from L929 cells, before being incubated for 7 days.

After 7 days, the unpolarised macrophages were dissociated with 0.02% EDTA solution (Merck E8008) and removed from the plates with a cell lifter (Corning CLS3008-100EA). Cells were counted with a haemocytometer (Marienfeld) before being centrifuged (400 rcf, 5 min, 21 °C) and resuspended in RPMI 1640, 5% FBS, 1% PenStrep to a density of $5x10^5$/mL.

2.3 Polarisation of primary human macrophages

Following isolation as described in section 2.2, macrophages were plated in 6-well plates (Corning 10578911) at $1.5x10^6$ (3 mL of $5x10^5$/mL) per well and allowed to re-adhere for 24 h. The next day medium was aspirated and either replaced with fresh RPMI 1640, 10% FBS, 1% PenStrep or the wells were washed 5 x with PBS and serum-free RPMI 1640 added. The macrophages were stimulated with LPS (0.2 ng/mL or 100 ng/mL, Enzo Life Sciences 581-010-L002) and IFN-γ (50 ng/mL, Peprotech 300-02), or IL-4 (20 ng/mL, Peprotech 200-04) and IL-13 (20 ng/mL, Peprotech 200-13) and incubated for 24 h.

2.4 siRNA knockdown by electroporation

Electroporation was carried out using an Amaxa 4D-Nucleofector X Unit (Lonza) with the P3 Primary Cell 4D-Nucleofector kit (Lonza V4XP-3024) for primary human macrophages and the SG Cell Line 4D-Nucleofector kit (Lonza V4XC-3024) for THP-1 cells. Primary human macrophages following isolation and polarisation with M-CSF as in section 2.2, or THP-1 cells, were counted, and resuspended in 4D-Nucleofector solution to a density of $1x10^7$/mL for primary macrophages and $2x10^7$/mL for THP-1 cells. Cells were split between treatment conditions and siRNA was added at a range of concentrations (50-200 nM). ON-TARGET Plus siRNA targeting *ADAM17* (Dharmacon SO-2792227G) was used, and non-targeting siRNA (Dharmacon D-001810-0X) was used as a negative control. Cells were transferred into cuvettes (100 μL per cuvette) which were placed into the nucleofector unit, and the appropriate programme run (DP-148 for primary macrophages, FF-100 for THP-1 cells). Following electroporation, cells were transferred into 1.9 mL pre-warmed RPMI 1640,

10% FBS, 1% PenStrep in 6-well plates to give a final density of $1x10^6$/mL for primary macrophages and $2x10^6$ for THP-1 cells, and incubated for 24-48 h.

2.5 Stimulation of THP-1 cells and primary human macrophages

Following isolation and polarisation as in section 2.2, primary macrophages were plated in 6-well plates at $1.5x10^6$ cells per well (3 mL of $5x10^5$/mL) and allowed to re-adhere for 24 h. THP-1 cells were plated in 6-well plates ($1x10^6$/2 mL per well), PMA (100 ng/mL, InvivoGen tlrl-pma) added, and incubated for 24 h. Conditioned media were aspirated and the cells washed 5 x with PBS to remove any residual serum which would interfere with trichloroacetic acid (TCA) precipitation and western blotting for LRP1. Fresh serum-free RPMI 1640 was added, and the cells stimulated with LPS (0.2 – 2000 ng/mL), IFN-γ (50 ng/mL), Dexamethasone (1 μM, Merck D4902), IL-1β (100 ng/mL, Peprotech 200-01B), IL-10 (10 ng/mL, Peprotech 200-10), and Ionomycin (100 nM – 50 μM, Tocris 1704) separately and in combination for a range of time points.

2.6 Inhibition of protease activity

Isolated primary macrophages were plated in 6-well plates at 3 mL ($5x10^5$/mL) per well and incubated for 24 h. THP-1 cells were plated in 6-well plates at 2 mL ($1x10^6$/mL) per well, PMA (100 ng/mL) added, and incubated for 24 h. The following day, conditioned media were aspirated from the cells and all wells washed 5 x with PBS. Serum-free RPMI 1640 was added along with the inhibitors (Table 2.1) and incubated for 1 h. Media were aspirated and replaced with fresh serum-free RPMI 1640 including the inhibitors, with or without LPS (0.2 or 100 ng/mL) and incubated for 6 h. For inhibitors which were dissolved in dimethyl sulfoxide [DMSO (Thermofisher Scientific 10103483)], the equivalent volume of DMSO was used as a control, and human immunoglobulin G (IgG) (BioXCell BE0092) was used to control for the D1(A12) blocking antibody at an equal concentration.

Table 2.1 Inhibitors of protease activity

Name	Supplier	Final concentration
AEBSF	Thermofisher Scientific 10357924	250 μM
CT1746	Gift from Celltech	100 μM
D1(A12) blocking antibody	Gift from Gill Murphy	0.33 μM
E-64	Thermofisher Scientific 16459007	10 μM

GI 254023X	Tocris 3995	5 μM
GM-6001	Enzo Life Sciences BML-EI300	100 μM
Pepstatin A	Tocris 1190	1 μM
PMSF	Tocris 4486	1 mM
TAPI-2	Tocris 6013	20 μM

2.7 Gel electrophoresis and immunoblotting

Proteins were precipitated from samples of conditioned media by the addition of TCA (MP Biochemicals 196057) to 5% v/v and overnight incubation at 4 °C. Samples were centrifuged (17150 rcf, 10 min, 4 °C), the supernatant discarded, and the pellets resuspended in 30 μL non-reducing sample buffer [100 mM tris(hydroxymethyl)aminomethane (Tris) HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 15% glycerol, 1% bromophenol blue]. Equal volumes (15 μL) of samples were loaded into the wells of 4-15% gradient sodium dodecyl sulfate-polyacrylamide electrophoresis gel (SDS-PAGE) gels (Bio-Rad 4561086) and ran at 180 V for 45 min. HiMark Pre-stained Protein Standard (Thermofisher Scientific LC5699) was ran alongside as a molecular weight marker. Proteins were transferred onto low fluorescence polyvinylidene difluoride (PVDF) membranes (Immobilon IPFL00005) with the Trans-Blot Turbo transfer system (Bio-Rad) using the high molecular weight programme, commercial buffer (20% transfer buffer, 20% ethanol), and the transfer pack (Bio-Rad 1704272).

Following transfer, membranes were stained for total protein using the REVERT 700 Total Protein Stain (LI-COR 926-11016) according to the manufacturer's instructions. The stain was imaged using an Odyssey CLx Imaging System (LI-COR) with Image Studio Ver 4.0 software (LI-COR). Membranes were blocked for 1 h at room temperature (RT) in blocking solution [5% dry milk (Marvel) in PBS] with constant shaking before being washed 3 x 5 min with washing solution [0.1% Tween-20 (Thermofisher Scientific 10113103) in PBS]. After washing, membranes were incubated with primary antibody (Table 2.2) diluted in Can Get Signal Immunoreaction Enhancer Solution 1 (2B Scientific TYB-NKB-101) overnight at 4 °C with constant shaking. Membranes were washed 3 x 5 min with washing solution before being incubated with a relevant secondary antibody (Table 2.2) diluted in Can Get Signal Immunoreaction Enhancer Solution 2 (2B Scientific TYB-NKB-10) for 1 h at RT with constant shaking. This was followed by a further 3 x 5 min washes with washing solution and then incubation with Clarity Western ECL Substrate (Bio-Rad 1705062) for 5 min at RT. Membranes were imaged using a G:BOX Chemi XRQ (Syngene) and images analysed using ImageJ (NIH) software.

Table 2.2 Antibodies used for western blotting

Antibody	Supplier	Dilution
Mouse anti-LRP1 (8G1)	Abcam ab20384	1:2000
Rabbit anti-LRP1 (N-terminal)	Sigma-Aldrich L2295	1:1000
Rabbit anti-mouse IgG HRP-conjugated	Fisher 10670264	1:5000
Goat anti-rabbit IgG HRP-conjugated	Abcam ab6721	1:5000

2.8 Enzyme-linked immunosorbent assay

TNF concentrations of conditioned media were measured using the OptEIA Human TNF enzyme-linked immunosorbent assay (ELISA) kit (BD Bioscience 555212) according to the manufacturer's protocol and final absorbance measured at 450 nm using an Omega Microplate Reader (BMG Labtech).

2.9 RNA isolation

RNA was isolated from cells using the RNeasy Mini Kit (QIAGEN 74106) according to the manufacturer's protocol and including the optional DNase step (QIAGEN 79254). Final RNA concentration was measured using a Nanodrop 2000/2000c spectrophotometer (Thermofisher Scientific) and the 260/280 and 260/230 ratios were recorded to indicate the purity of the samples.

2.10 cDNA synthesis and qRT-PCR

40-400 ng of isolated RNA was used to synthesise complementary DNA (cDNA) using the qPCRBIO cDNA Synthesis Kit (PCR Biosystems PB30.11-10) according to the manufacturer's protocol. cDNA synthesis was carried out using the T100 Thermal Cycler (Bio-Rad) to incubate samples at 42 °C for 30 min, followed by 85 °C for 10 min.

This cDNA was diluted 1 in 4 using RNase-free water and quantitative real time reverse transcription-polymerase chain reaction (qRT-PCR) carried out using a LightCycler 480 II (Roche) and either TaqMan Fast Advanced Mastermix (Thermofisher Scientific 4444556) and TaqMan primers (Thermofisher Scientific, Table 2.3), or qPCRBIO SyGreen Mix (PCR Biosystems PB20.11-05) and KiCqStart SYBR Green primers (Merck, Table 2.4) to measure the expression of target genes. qRT-PCR using Taqman primers was done using the following conditions: 60 °C for 30 s, 95 °C for 5 min, 45 cycles of 95 °C for 15 s, 60 °C

for 60 s, and 40 °C for 30 s. When using the SYBR Green primers, the conditions were: 95 °C for 5 min, 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. In order to validate that the SYBR Green primers only amplified one target, melt curves were done using these conditions: 95 °C for 5 s, 65 °C for 1 min and 97 °C with 5 acquisitions per °C. Delta cycle threshold (Ct) values were calculated in relation to the expression of a housekeeper gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)*. Fold change in gene expression was calculated using the delta delta Ct method. qRT-PCR was done using SyGreen Mix and SYBR Green primers unless otherwise specified.

Table 2.3 Taqman human primers

Gene name	**Primer code**
ADAM8	Hs00923290_g1
ADAM9	Hs00177638_m1
ADAM10	Hs00153853_m1
ADAM12	Hs01106101_m1
ADAM15	Hs00187052_m1
ADAM17	Hs01041915_m1
ADAM19	Hs00224960_m1
ADAM20	Hs010831787_s1
ADAM28	Hs00248020_m1
ADAM30	Hs00253969_s1
ADAM33	Hs00905552_m1
GAPDH	Hs02758991_g1
LRP1	Hs00233856_m1
TIMP-3	Hs00165949_m1

Table 2.4 KiCqStart SYBR Green human primers

Gene name	**Forward sequence 5'-3'**	**Reverse sequence 5'-3'**
ADAM8	CAATGCAGAGTTCCAGATG	TTTCTGATATAGCTTGTCCAC
ADAM9	AGGTATGACATGATGGGAAG	CATACTATCCAAGTAGTTTGCC
ADAM10	CACATGATTCTGGAACAGAG	GTTGTTAAGTTTGTCCCCAG

ADAM12	AGTTTTACAGACCACTGAAC	TAGAAGCTTCATCTTCCTCC
ADAM15	AGCTAATACTCGGGGAAATG	GTCCCATTCACATCTATTGTC
ADAM17	CAGATTCGCATTCTCAAGTC	CTAGCAACATCTTCACATCC
ADAM19	GTTTCAGAAGAATCGACGAG	GACCAGAGGGTAGAATATGG
ADAM20	AATGGAACACTGAAAGACAC	TGTCTAGAGCAGAAGAGAAC
ADAM28	ATCCAGTGTCTACACCTAAG	CTTGAGTCGAGAGCAAATAC
ADAM30	AACTGTAGCATTGGACTTTG	CCATCCTGCTTATAAACGTC
ADAM33	CCCAGGATACATAGAAACCC	GCAGATAATAGCTGGCATTC
CD14	GATTACATAAACTGTCAGAGGC	TCCATGGTCGATAAGTCTTC
CD16	CGGATATCTTTGGTGACTTG	AACTAGAAGTAGCAGAGCAG
CD68	GAGACTTTCATTTCCTCCTTTC	TTTTGTGAGGACAGTCATTC
CD80	AGGAGGAATGAGAGATTGAG	GACCTTCAGATCTTTTCAGC
CD206	AAATTTGAGGGCAGTGAAAG	GGATTTGGAGTTTATCTGGTAG
CD209	CAGCTCGTCGTAATCAAAAG	TTAGATCTGAAAGTCCCATCC
GAPDH	ACAGTTGCCATGTAGACC	TTGAGCAACAGGGTACTTTA
IL1B	GCCTTCAAGGAAAAGAATCTG	GGATCTACACTCTCCAGC
iRHOM1	GGTCCTCTTCTACGTCTATC	GTTCGTACTTCTCACAGAAC
iRHOM2	CTGATGATGTCTTTGAGTCC	TACTCCTTCAGAGGGATTTG
LRP1	ACATATAGCCTCCATCCTAATC	GCTTATACCAGAATACCACTC
MT1-MMP	ATGGCAAATTCGTCTTCTTC	CGTTGAAACGGTAGTACTTG
TIMP-3	CATGTGCAGTACATCCATAC	AGGTGATACCGATAGTTCAG
TNF	AGGCAGTCAGATCATCTTC	TTATCTCTCAGCTCCACG
tPA	GGAATTCCATGATCCTGATAG	TCCGGCAGTAATTATGTTTG
TSPAN5	CAGAAGATGTCATCAACACTC	ACGATGGTTAAATTGTCCTG
TSPAN10	GCAGAACCTGTACTTTAACTG	CAGGTACACCACTCTCTG
TSPAN14	AGTGTGGATATGATGTCAGG	ATGCCAAATATCTGCAACAG
TSPAN15	CAGAAAAAGTTCAAGTGCTG	GCTCCTTGTCGATAGTTTTG
TSPAN17	CTCAACCTCTTCATCAACAAC	CAGTATTCCTGAGCAAAGTC
TSPAN33	ATTTCAACTGCTCAGAAGAC	CCATTGGTGTAGATGACTTTG

2.11 MTS cell proliferation assay

At the desired time point, media were removed from cells cultured in 96-well flat bottom plates (Corning 3599) and 120 μL of fresh RPMI 1640, 10% FBS, 1% PenStrep, 16.7% 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega G3580) was added to each well. The plates were incubated at 37 °C, 5% CO_2 for 1 h and absorbance at 490 nm was measured using an Omega Microplate Reader (BMG Labtech). A standard curve was constructed by plating a serial dilution of cells from 200000 to 0 in triplicate and plotting cell number versus absorbance at 490 nm.

2.12 Statistical analysis

Data were analysed and graphs created using GraphPad Prism Version 9.4.1 for Windows (GraphPad Software). Where $n > 4$, data was first tested for normality using a Shapiro-Wilk test. This was necessary to test whether the data was normally distributed or not as this would affect what statistical tests were then used to analyse the significance of any differences. Where this test was positive, parametric statistical tests were used to compare treatment groups to control groups. If the data was not normally distributed, non-parametric statistical tests were used. For data which was presented as a ratio or percentage of the control, non-parametric tests were always used. This was because the control values were all set to 1 or 100 %, and so could not be normally distributed. Where $n < 4$, normality cannot be tested as there are not enough data points to analyse the distribution. Therefore, non-parametric statistical tests were done. The specific test done for each data set is given in the figure legends. For any data where $n < 3$, no statistical tests could be done. A p-value of less than 0.05 was considered statistically significant.

Chapter 3: Investigating LRP1 shedding in human monocytic cell lines

3.1 Introduction

In order to investigate how LRP1 shedding is regulated in human macrophages, I needed a tractable model system in which LRP1 shedding could be specifically and sensitively quantified. In particular, I wanted to investigate LRP1 shedding in response to pro-inflammatory stimuli such as LPS or IFN-γ which have previously been demonstrated to stimulate LRP1 shedding (Gorovoy et al., 2010; Schubert et al., 2019). I first decided to test whether two monocytic cell lines could be used for this purpose.

The THP-1 cell line is a human monocytic leukaemia cell line which differentiates to macrophage-like cells upon the incubation with PMA (Tsuchiya et al., 1980) while the U937 cell line is a human pro-monocytic myeloid leukaemia cell line which can also differentiate to macrophage-like cells with PMA treatment (Sundström & Nilsson, 1976). U937 cells originate from tissue, so are more mature than THP-1 cells which originate from peripheral blood (Chanput et al., 2014). Furthermore, THP-1 cells have very low levels of *CD14* expression (Bosshart & Heinzelmann, 2004). This is a transmembrane protein which is involved in the LPS-receptor complex (Park et al., 2009) and so THP-1 cells do not respond well to LPS stimulation.

When PMA-differentiated, both cell lines are a simplified version of human macrophages but can be a very useful and convenient way of investigating cellular processes (Qin, 2012; Tedesco et al., 2018). For example, THP-1 cells have been used to characterise ADAM17-dependent TNF release (Doedens & Black, 2000). However, as both cell lines are derived from tumours, they are different to circulating human monocytes and so it was necessary to investigate which cell line might be most appropriate for my research.

3.2 Optimisation of western blotting protocol for the detection of shed LRP1 in the conditioned media of human macrophages

To investigate the shedding of LRP1 I decided to use western blotting to analyse the amount of LRP1 shed into the conditioned media. Other methods such as RAP-ligand blotting (Gaultier et al., 2008; Gorovoy et al., 2010) and flow cytometry (Schubert et al., 2019) have been used previously, but western blotting LRP1 in the conditioned media should give the most accurate picture of shedding as it is a direct measure of just the shed LRP1 and is not obscured by potential changes in intracellular or cell surface levels.

I tested two primary antibodies so that I could optimise the sensitivity of the western blot. The first was a monoclonal mouse antibody (clone 8G1) which recognises an epitope at the N-terminus of the α-chain and has been widely used in the literature (Boyé et al., 2017;

Scilabra et al., 2013; Selvais et al., 2011). The second was a polyclonal antibody raised in rabbits using a synthetic peptide corresponding to amino acids 188-201 of human LRP1 and which also has been used previously (Gorovoy et al., 2010; Rauch et al., 2020). As a positive control, I used 30 ng of purified full-length human LRP1 (Yamamoto et al., 2014) purchased from BioMac. Both antibodies detected bands at the same molecular weights (Figure 3.1) but the 8G1 monoclonal antibody produced a much stronger signal. Therefore, I decided to use this 8G1 antibody for all future western blots of LRP1.

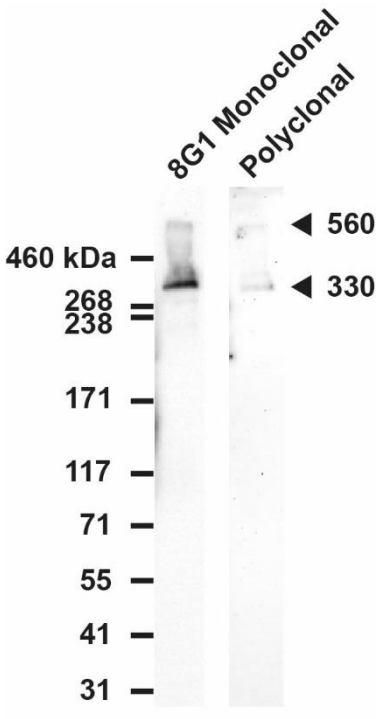

Figure 3.1 8G1 monoclonal anti-LRP1 antibody was more sensitive than the polyclonal antibody.

30 ng of purified human LRP1 was electrophoresed on an SDS-PAGE gel and immunoblotted for LRP1 using two different primary antibodies. Molecular weights of bands detected (◀) were calculated using the HiMark prestained protein ladder to be 330 kDa and 560 kDa.

Because I planned to western blot conditioned media, it was necessary to concentrate the media before electrophoresis. I planned to do this by adding TCA to precipitate proteins from the media. To ensure reproducible TCA precipitation, previous studies (Doherty et al., 2016) have included 0.1% FBS in the media to “seed” the precipitation. I investigated whether FBS contained protein(s) that could be detected by the anti-LRP1 antibody and found that the antibody detected several bands, the strongest of which was roughly 170 kDa (Figure 3.2A). This band was still detectable when the FBS concentration was dropped to 0.1% (Figure 3.2B). To see whether this signal would interfere with the detection of LRP1

shed from cells, I also tested conditioned media from PMA-differentiated-THP-1 cells which had been stimulated with LPS (100 ng/mL) for 24 h in the absence of serum.

The bands detected by the anti-LRP1 antibody in serum-free conditioned media from THP-1 cells were considerably weaker than the bands in the 0.1% FBS sample, and some of the lower molecular weight bands overlapped with the bands in the 0.1% FBS sample. This meant that any FBS present in the conditioned media would interfere with the analysis of shed LRP1 and so all experiments where western blotting was used to analyse LRP1 shedding would need to be done without serum.

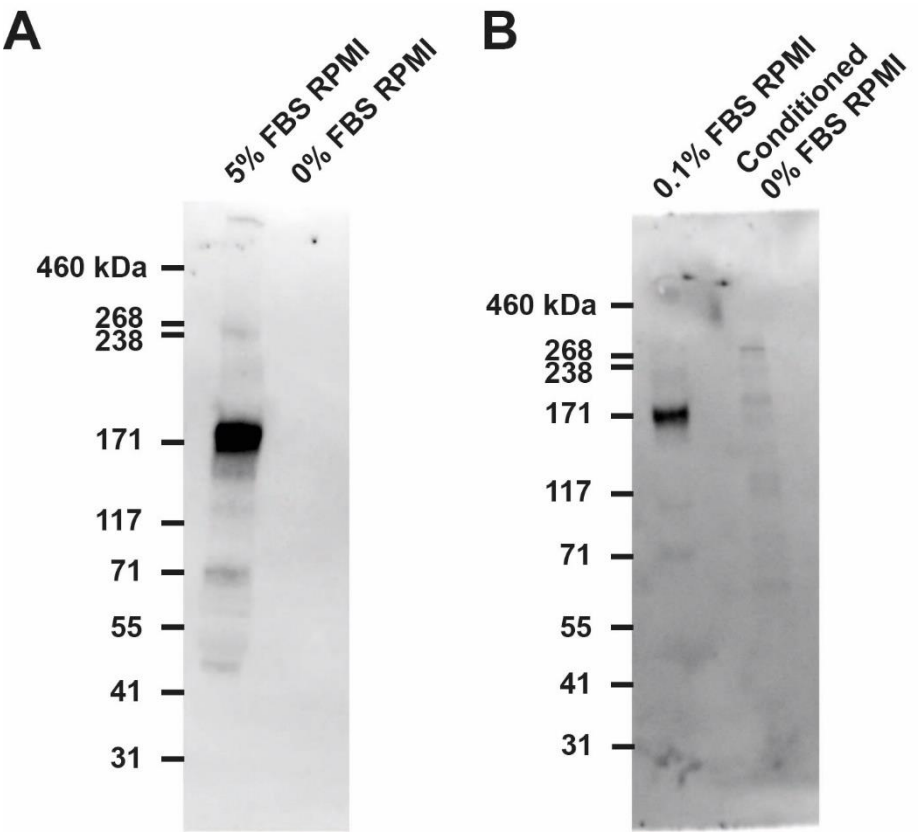

Figure 3.2 Anti-LRP1 antibody detected bands present in FBS at both 5% and 0.1% which interfered with the detection of shed LRP1.

(A) RPMI 1640 containing either 5% FBS or 0% FBS was diluted in a 1:1 ratio with SDS sample buffer before analysis by SDS-PAGE and immunoblotting for LRP1. (B) RPMI 1640 containing 0.1% FBS was diluted in a 1:1 ratio with SDS sample buffer. $1x10^6$ THP-1 cells were treated with PMA (100 ng/mL) for 24 h followed by LPS (100 ng/mL) for 24 h in the absence of FBS. Conditioned media were collected and concentrated with TCA. Both samples were analysed by SDS-PAGE and immunoblotting for LRP1.

The cell lysate contains not only cell surface LRP1, but also intracellular LRP1, either as it is synthesised or after endocytosis and recycled to the cell membrane. Therefore, western blotting the cell lysate for LRP1 is an inaccurate method of investigating functional LRP1 on

the cell surface. However, when western blotting the conditioned media there is not an appropriate housekeeper protein, such as actin, to control for protein loading. One method of controlling for sample loading is to normalise western blot bands against a total protein stain conducted prior to blocking the PVDF membrane.

I tested the Revert Total Protein Stain using a pooled sample loaded equally into every lane of an SDS-PAGE gel. This showed that there was very little variation in signal across the membrane (Figure 3.3) and that the Revert Total Protein Stain was a consistent method of relative total protein quantification. Therefore, I used this method to normalise all further LRP1 western blots.

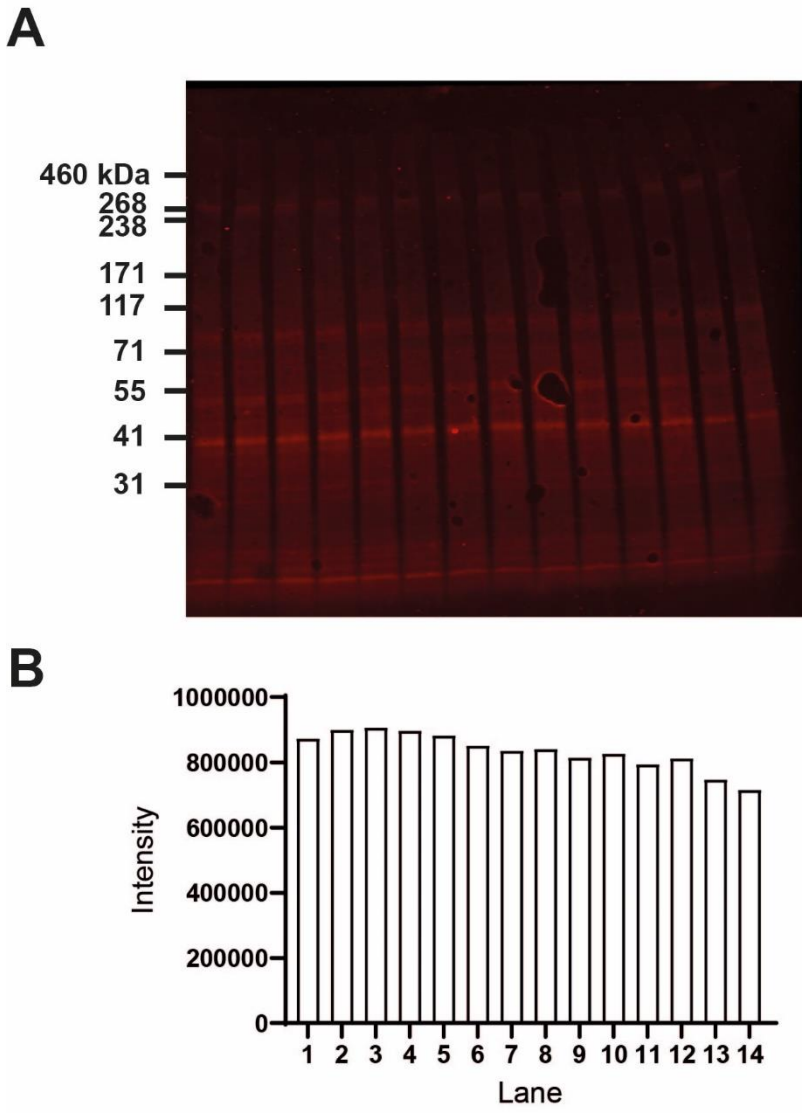

Figure 3.3 Revert Total Protein Stain gave consistent signal across the entire membrane with very little variation.

10 µL of identical sample was electrophoresed in every lane of an SDS-PAGE gel and transferred to PVDF membrane. The membrane was stained with Revert Total Protein Stain (A) and lane intensities quantified (B).

3.3 Investigating the effect of LPS stimulation on PMA-differentiated THP-1 and U937 cell lines

PMA-differentiated THP-1 and U937 are the most commonly used cell line models to study human macrophages but I wanted to investigate which cell line would be most appropriate for investigating LRP1 shedding.

I first profiled the expression of the active *ADAMs* (Klein & Bischoff, 2011) in THP-1 and U937 cells without any differentiation or stimulation. I found that *ADAM17*, a key candidate LRP1 sheddase, was similarly expressed in both cell types (Figure 3.4). This pattern was the same for the majority of the other *ADAMs* except for *ADAM15,* where expression was not detected in THP-1 cells compared with a delta CT value of 7 in U937 cells.

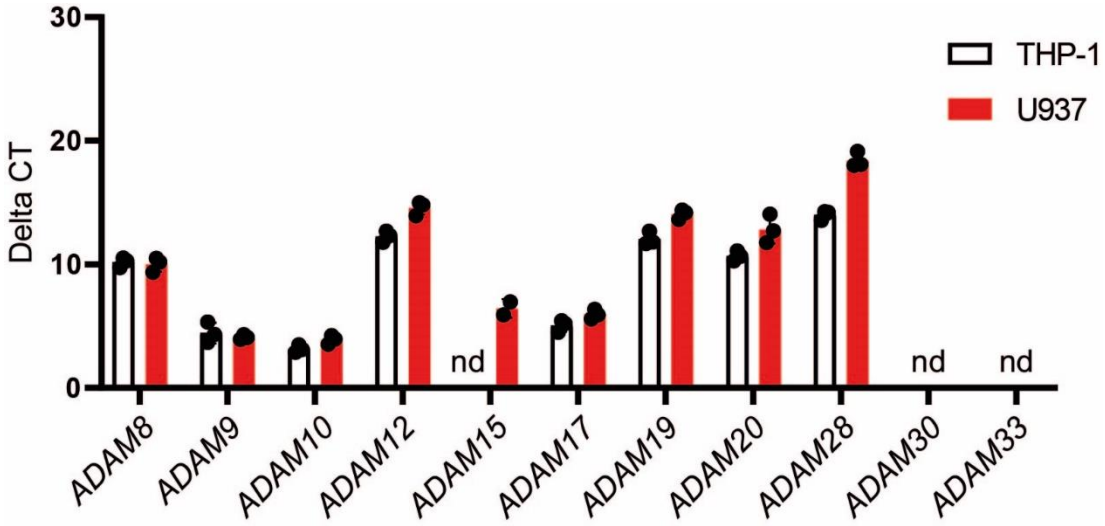

Figure 3.4 THP-1 and U937 cells had a similar pattern of expression of *ADAMs* except for *ADAM15*.
$1x10^6$ THP-1 and U937 cells were cultured for 24 h in RPMI 1640 with 10% FBS. RNA was isolated and reverse transcribed to cDNA. Delta CT values of *ADAMs* were calculated by qRT-PCR relative to the housekeeper *GAPDH* using Taqman mastermix and primers (mean ± standard deviation (SD), 3 independent replicates).

I next investigated how the two cell lines responded to TLR activation which is one of the primary triggers of pro-inflammatory cytokine production (Akira et al., 2006). As it is hypothesised that the shedding of LRP1 is involved in terminating the release of TNF (Schubert et al., 2019), I needed to be able to stimulate TNF release in any model I used to investigate LRP1 shedding.

I tested the bacterial-derived β-glucan curdlan (10 µg/mL), the yeast-derived β-glucan zymosan (10 µg/mL), and LPS purified from *E. coli* (100 ng/mL). The two β-glucans interact

with TLR2 (de Graaff et al., 2021) while the LPS receptor is TLR4 (Lu et al., 2008). I treated the PMA-differentiated cells from each cell line with the TLR activators for 24 h and measured TNF release by ELISA (Figure 3.5A). I observed the same pattern of activation in both cell lines however U937 cells produced much more TNF than THP-1 cells, in line with previous reports of limited TNF release from THP-1 cells in response to LPS (Bosshart & Heinzelmann, 2004). Curdlan had no effect on TNF release, however both zymosan and LPS stimulated TNF release. LPS stimulated the largest release of TNF in both cell lines.

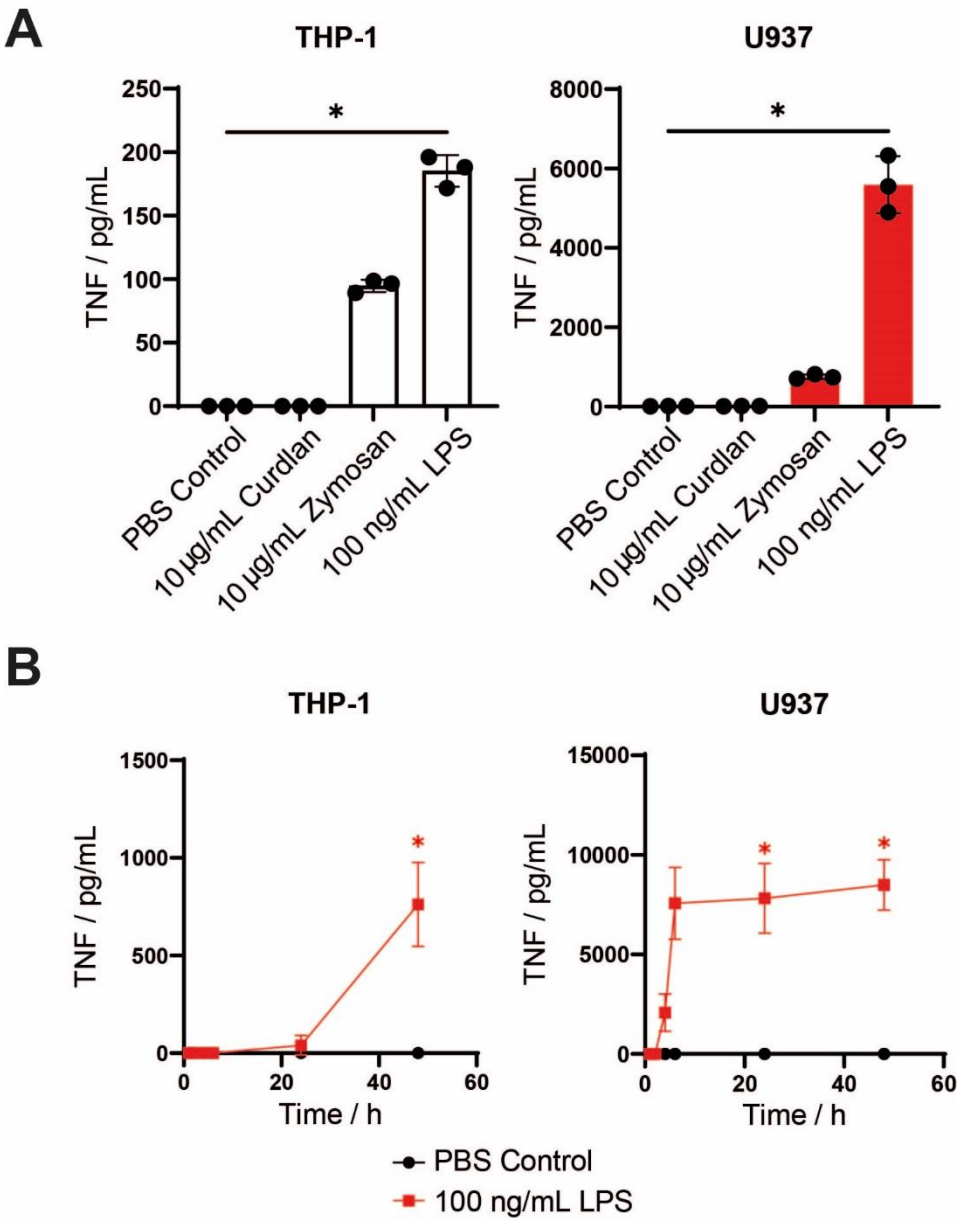

Figure 3.5 TNF release was stimulated most effectively by LPS in U937 cells.
(A) 1x10^6 THP-1 and U937 cells were treated with PMA (100 ng/mL) for 24 h. They were then treated with curdlan (10 µg/mL), zymosan (10 µg/mL), LPS (100 ng/mL), or PBS as a control for a further 24 h in the presence of serum. TNF concentration in conditioned media was analysed by ELISA. Treatment conditions were compared to the PBS control using a Kruskal-Wallis and corrected for multiple comparisons with Dunn's test (mean ± SD, 3

independent replicates, * $p < 0.05$). (B) $1x10^6$ THP-1 and U937 cells were treated with PMA (100 ng/mL) for 24 h followed by LPS (100 ng/mL) or PBS for 48 h. TNF concentration in conditioned media at 1, 2, 4, 6, 24, and 48 h after LPS addition was analysed by ELISA. LPS-stimulated conditions were compared to the PBS control using multiple Mann-Whitney U tests (mean ± SD, 3 independent replicates, * $p < 0.05$).

Next, I tested how TNF was released over a 48 h time period in response to LPS stimulation (Figure 3.5B). I found that in THP-1 cells, TNF release was a slow response with the majority of TNF being released between 24 and 48 h after stimulation. In comparison, TNF release from U937 cells was detectable by 4 h and had reached a plateau by 6 h which was maintained for 48 h.

For my research, the most important aspect that I needed to test for each cell line was whether I could detect shed LRP1 in the conditioned media by western blotting. It has been previously reported that LPS stimulates the shedding of LRP1 from human and murine macrophages (Gorovoy et al., 2010; Schubert et al., 2019), and so I tried to demonstrate the same effect in both THP-1 and U937 cells.

I first tested whether I could detect LPS-stimulated LRP1 shedding from PMA-differentiated THP-1 cells after 6 h of LPS stimulation (Figure 3.6A). I was able to detect shed LRP1 in the media of both the LPS-stimulated cells and the PBS-treated control cells, however I was unable to detect any LPS-stimulated LRP1 shedding at this time point.

I stimulated PMA-differentiated U937 cells with LPS (100 ng/mL) for 48 h and immunoblotted the concentrated conditioned media at 1, 2, 4, 6, 24, and 48 h time points (Figure 3.6B). Unfortunately, I could not detect any LRP1 in the conditioned media of U937 cells at any time point of LPS-stimulation.

The highest bands I observed in THP-1 conditioned media were at around 330 kDa (marked by ◄) which while not the expected size for the α-chain of LRP1 (515 kDa) is consistent with the strongest band on the purified LRP1 blot (Figure 3.1). Bands at this molecular weight have also been reported in the literature using this antibody (Selvais et al., 2011). Therefore, I was confident that the bands I observed at this molecular weight were indeed shed LRP1. Consequently, I decided to use THP-1 cells rather than U937 cells for further investigation of LRP1 shedding.

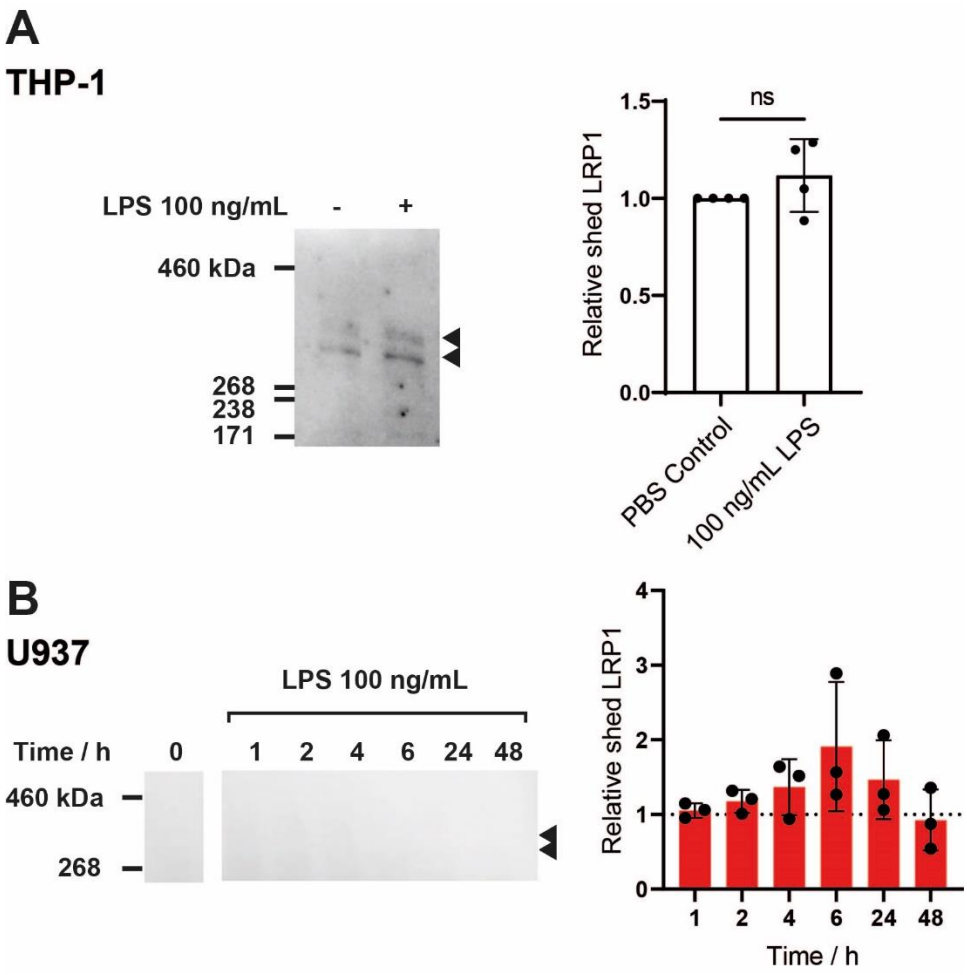

Figure 3.6 LRP1 was detected in the conditioned media from THP-1 cells but LRP1 shedding was not stimulated by LPS.

(A) $2x10^6$ THP-1 cells were treated with PMA (100 ng/mL) for 24 h followed by LPS (100 ng/mL) or PBS for 6 h. Conditioned media were collected and concentrated with TCA and analysed by SDS-PAGE and immunoblotting for LRP1. Bands (◄) were quantified and normalised against a Revert Total Protein Stain and shed LRP1 levels calculated relative to the unstimulated control. Data were analysed using a paired t-test (mean ± SD, 4 independent replicates). (B) $1x10^6$ U937 cells were treated with PMA (100 ng/mL) for 24 h followed by LPS (100 ng/mL) or PBS for 48 h. Conditioned media were collected at 1, 2, 4, 6, 24, and 48 h after LPS addition, concentrated with TCA and analysed by SDS-PAGE and immunoblotting for LRP1. Bands (◄) were quantified and normalised against Revert Total Protein Stain and shed LRP1 levels calculated relative to the unstimulated control at 0 h. Each time point was compared to the 0 h time point using a Kruskal-Wallis and corrected for multiple comparisons with Dunn's test (mean ± SD, 3 independent replicates).

3.4 The effects of inflammatory stimulation on LPS release and LRP1 shedding in THP-1 cells

Following the conclusion that I could not detect LPS-stimulated shedding of LRP1 from PMA-differentiated THP-1 cells, I investigated whether other pro- or anti-inflammatory cytokines might influence LRP1 shedding. I treated PMA-differentiated THP-1 cells with several pro-inflammatory stimuli, LPS (100 ng/mL), IL-1 (100 ng/mL), and IFN-γ (50 ng/mL). I also tested the anti-inflammatory corticosteroid dexamethasone (1 μM) and the anti-inflammatory cytokine IL-10 (10 ng/mL) alone and in combination with LPS. I first measured TNF release in response to these stimuli and found that IFN-γ stimulation produced the highest level of TNF release, followed by the combination of LPS and IL-10 (Figure 3.7A). The remaining conditions all did not significantly stimulate TNF release.

When I immunoblotted the conditioned media, I was able to detect LRP1 in all conditions but no significant changes in LRP1 shedding were evident (Figure 3.7B and C). Since Gorovoy *et al.* (2010) showed that both LPS and IFN-γ could stimulate LRP1 shedding from murine macrophages, I decided to investigate the effects of IFN-γ further.

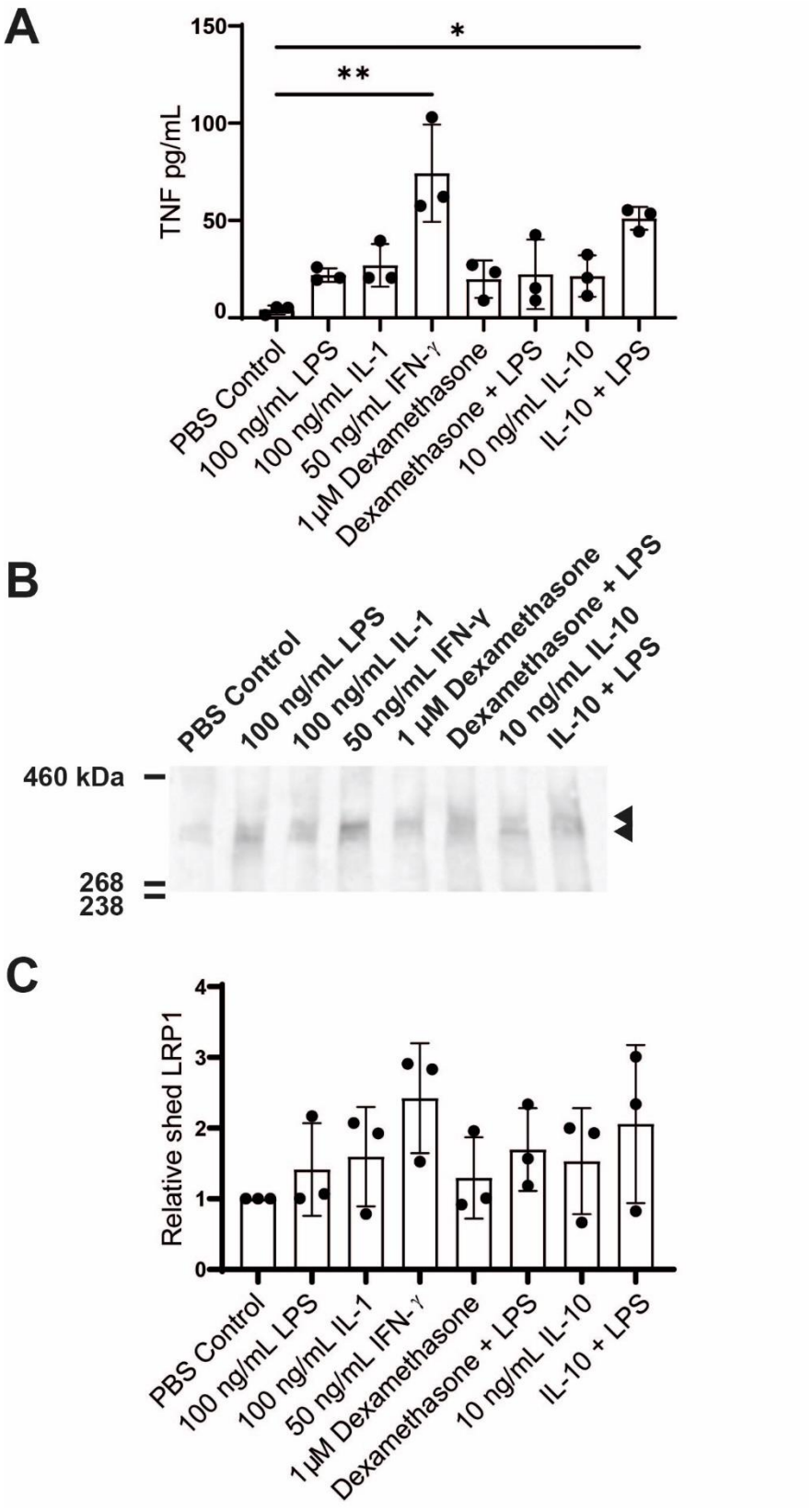

Figure 3.7 IFN-γ caused the greatest release in TNF from THP-1 cells, but none of the tested cytokines significantly stimulated LRP1 shedding at 24 h.

2x10^6 THP-1 cells were treated with PMA (100 ng/mL) for 24 h, and then with LPS (100 ng/mL), IL-1 (100 ng/mL), IFN-γ (50 ng/mL), dexamethasone (1 μM), IL-10 (10 ng/mL), combinations of these cytokines, or PBS as a control for a further 24 h. (A) TNF

concentration in conditioned media was analysed by ELISA. Treatment conditions were compared to the PBS control using a Kruskal-Wallis and corrected for multiple comparisons with Dunn's test (mean ± SD, 3 independent replicates, * $p < 0.05$, ** $p < 0.01$). (B) Conditioned media were collected and concentrated with TCA before analysis by SDS-PAGE and immunoblotting for LRP1 (A). Bands (◄) were quantified and normalised against a Revert Total Protein Stain and shed LRP1 levels calculated relative to the PBS control (B). Treatment conditions were compared to the PBS control using a Kruskal-Wallis and corrected for multiple comparisons with Dunn's test (mean ± SD, 3 independent replicates).

I first treated PMA-differentiated THP-1 cells with IFN-γ or IFN-γ in combination with dexamethasone for 24 h and measured TNF release by ELISA at 2, 6, and 24 h (Figure 3.8A). I found that IFN-γ only significantly stimulated TNF release at 24 h and this effect was reduced by co-treatment with dexamethasone but not completely abolished. This demonstrated that dexamethasone was indeed having an anti-inflammatory effect on the cells and so any lack of effect on LRP1 shedding was not due to the cells not responding to dexamethasone.

I also tested the effect of IFN-γ in combination with LPS on TNF release and observed an additive effect of the two cytokines. However, I only did one replicate of this so was unable to do any statistical tests on the data.

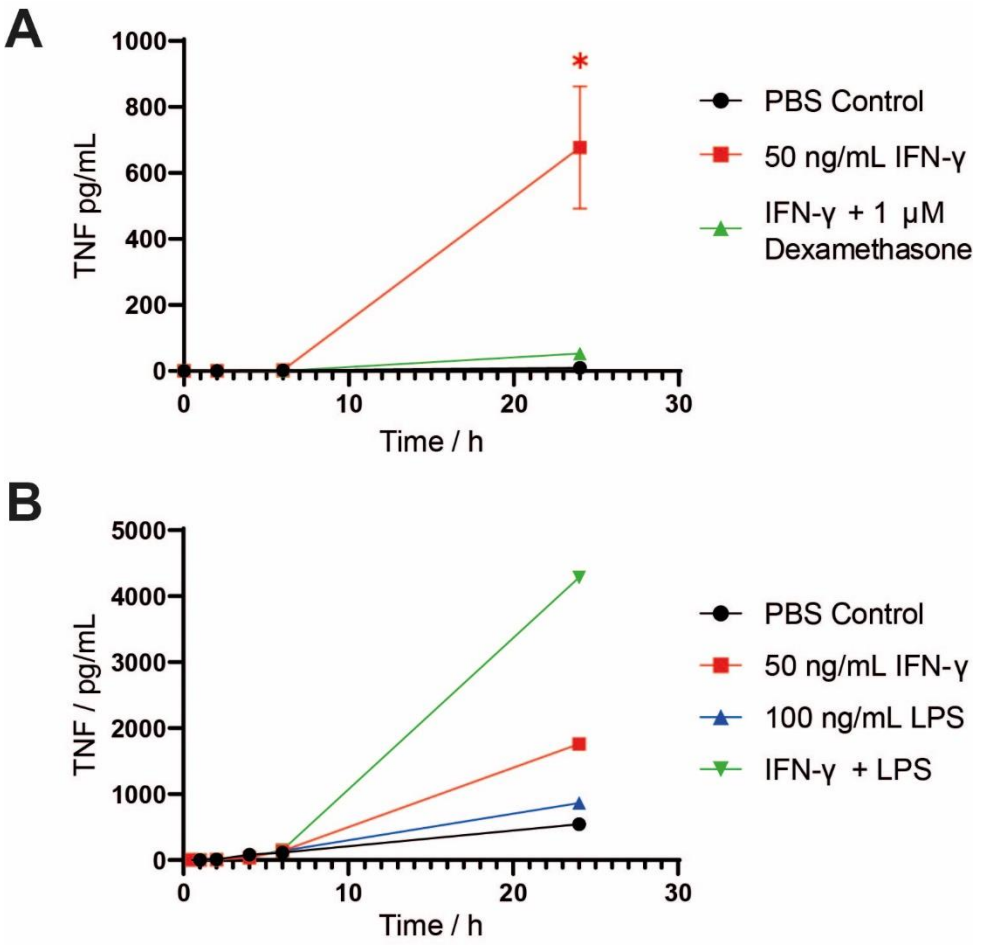

Figure 3.8 IFN-γ stimulation of TNF release was reduced by dexamethasone and increased by LPS.

(A) $2x10^6$ THP-1 cells were treated with PMA (100 ng/mL) for 24 h, and then treated with IFN-γ (50 ng/mL), IFN-γ and dexamethasone (1 μM), or PBS as a control for a further 24 h. TNF concentration in conditioned media at 2, 6, and 24 h after stimulant addition was analysed by ELISA. Treatment conditions were compared to the PBS control at each time point using multiple Mann-Whitney U tests (mean ± SD, 3 independent replicates, * $p < 0.05$). (B) $2x10^6$ THP-1 cells were treated with PMA (100 ng/mL) for 24 h. They were then treated with IFN-γ (50 ng/mL), LPS (100 ng/mL), a combination of the two, or PBS as a control for 24 h. TNF concentration in conditioned media at 1, 2, 4, 6, and 24 h after stimulant addition was analysed by ELISA (1 replicate).

I then immunoblotted the conditioned media from PMA-differentiated THP-1 cells after a time course of IFN-γ stimulation (Figure 3.9). I found that treatment with IFN-γ had no significant effect on the levels of shed LRP1 in the conditioned media at any of the time points I measured.

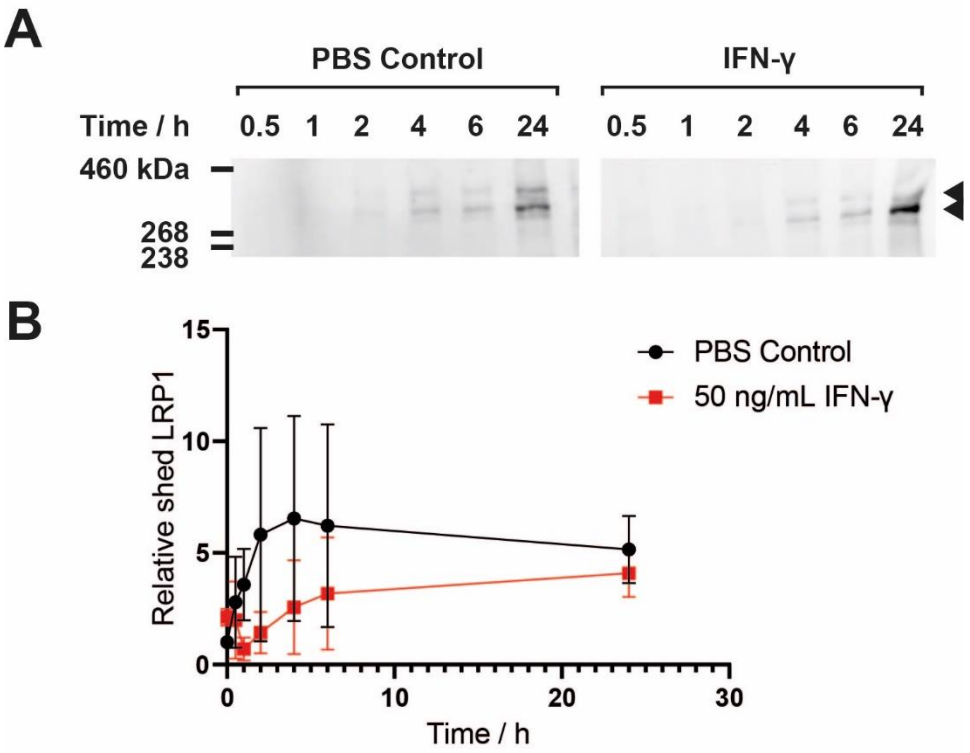

Figure 3.9 IFN-γ treatment had no significant effect on LRP1 shedding from THP-1 cells at the time points tested.

(A) 2x10^6 THP-1 cells were treated with PMA (100 ng/mL) for 24 h and then treated with IFN-γ (50 ng/mL) or PBS as a control for a further 24 h. Conditioned media were collected 0.5, 1, 2, 4, 6, and 24 h after IFN-γ or PBS treatment, and concentrated with TCA before analysis by SDS-PAGE and immunoblotting for LRP1. Bands (◄) were quantified and normalised against a Revert Total Protein Stain and shed LRP1 levels calculated relative to the PBS control at 0 h (B). IFN-γ treated conditions were compared to the PBS control at each time point using multiple Mann-Whitney U tests (mean ± SD, 3 independent replicates).

3.5 Discussion

Several methods have previously been used to investigate the shedding of LRP1. RAP-ligand blotting has greater sensitivity than immunoblotting (Gaultier et al., 2008; Gorovoy et al., 2010; Liu et al., 2009a). This method uses a recombinant fusion protein of RAP with glutathione S-transferase as the detection step, followed by an anti-glutathione-S-transferase (GST) antibody and a horseradish peroxidase (HRP)-linked secondary antibody. However, RAP can bind to all members of the LDL receptor family so will detect all of those as well as LRP1 (Medh et al., 1995; Williams et al., 1992), so this method is not as specific as using a primary antibody for detection.

An alternative method is to use flow cytometry to quantify the cell surface levels of the LRP1 α-chain compared to the levels of the β-chain (Schubert et al., 2019). This indirect method of measuring the shedding of LRP1 was sensitive enough to detect LPS-stimulated shedding of LRP1 at 8 h but the change was small and insufficient to detect LRP1 shedding in the basal, unstimulated condition. This method also gives no molecular weight information, so it does not show whether shed LRP1 is fragmented or not.

LRP1 shedding can also be measured using an ELISA to measure the concentration of LRP1 in the conditioned media. However, like the flow cytometry, this does not show the molecular weight of the shed LRP1 which is important information. Furthermore, the commercial LRP1 ELISA kits are very expensive.

Measuring the levels of LRP1 in the conditioned media by western blotting is thus the most accurate and direct method of investigating LRP1 shedding. It allows for quantification of the levels of LPS-stimulated LRP1 shedding and also enabled detection of LRP1 shedding in the basal, unstimulated state. This method is more specific than RAP-ligand blotting as the primary antibodies which are commonly used are specific and will only bind to LRP1. Western blotting also shows the molecular weight of detected bands, which enables investigation of whether LRP1 is fragmented post shedding as has been previously reported (Gaultier et al., 2008; Scilabra et al., 2013; Selvais et al., 2011).

Unfortunately, the primary antibodies used to directly immunoblot conditioned media are not particularly sensitive and the band for the full-length LRP1 α-chain (515 kDa) is often very faint compared with bands for LRP1 fragments. This could make analysis of changes in LRP1 shedding difficult using traditional western blotting if the cells used do not shed much LRP1.

I decided to immunoblot the conditioned media with an anti-LRP1 primary antibody to directly measure the levels of LRP1 shedding. I chose the monoclonal mouse antibody (clone 8G1) as of the two antibodies I tested, this gave the strongest signal when I immunoblotted purified full-length LRP1. The purified LRP1 that I used should be roughly 600 kDa in size as it consists of both the α- and β-chain subunits. Both antibodies detected bands at the same molecular weight (approximately 330 and 560 kDa) with the strongest intensity band at 330 kDa potentially arising from fragmentation of the protein. The higher molecular weight band (approximately 560 kDa) may be full length LRP1, as this band runs above the largest marker of the ladder so its size cannot be accurately determined.

I found that FBS contains shed LRP1, in line with previous reports of LRP1 shedding into serum (Quinn et al., 1997). This meant that serum-free conditions were required for samples that I wanted to western blot for LRP1. For all future experiments, I thus washed cells multiple times with PBS to remove all traces of FBS which was present in the growth media,

before adding serum-free RPMI 1640 for the time period under investigation. TCA precipitation of this medium was reproducible despite the lack of serum.

The final step of optimisation was to control for protein loading. To control for this as much as possible prior to SDS-PAGE and western blotting, in all experiments I used an equal number of cells in each condition, grown in an equal volume of media, which was then precipitated by adding an equal percentage of TCA and resuspended in an equal volume of SDS sample buffer. Equal volumes were then loaded onto each lane of the SDS-PAGE gel, and the blot stained with the fluorescent Revert Total Protein Stain. LRP1 band intensities were normalised to the amount of protein in each lane.

In all of the western blots of conditioned media that I did, the highest molecular weight LRP1 bands that I observed were the two bands at roughly 330 kDa. Some lower molecular weight bands were present in some western blots demonstrating the fragmentation of LRP1 previously observed with western blotting (Gaultier et al., 2008; Scilabra et al., 2013; Selvais et al., 2011) but they were generally much weaker. The relative strength and consistency between blots of the double band at 330 kDa, combined with the fact that this was the molecular weight at which the purified full-length LRP1 produced its strongest band led me to conclude that these bands corresponded with shed LRP1. In order to confirm that the bands were indeed LRP1 I would have liked to perform N-terminal sequencing. This would also have allowed investigation into where LRP1 is fragmented post shedding. Unfortunately, due to time constraints, this was not possible.

Now that I had a method of measuring LRP1 levels in conditioned media, I started to investigate which of the cell lines, THP-1 or U937, might make a better model system for my research.

There are a vast number of different protocols for PMA-differentiation of THP-1 and U937 cells to a macrophage-like phenotype (Baxter et al., 2020; Chanput et al., 2014; Kurihara & Furue, 2013). I used 100 ng/mL PMA for 24 h as this was a widely reported concentration and by this time point, all the cells were adhered to the cell culture plate. This was a quick, visual, confirmation of differentiation as the monocyte-like cells did not adhere.

Both cell lines had very similar expression profiles of the *ADAMs* with the exception of *ADAM15,* which was not detected in THP-1 cells. This did not rule THP-1 cells out as a model system as ADAM15 is not known to be involved in TNF release, and while it does have a role in inflammation (Mosnier et al., 2006) is not one of the metalloproteases hypothesised to be responsible for LRP1 shedding.

THP-1 cells released much less TNF and at a slower rate in response to TLR activation than U937 cells, although the pattern of responses to the different stimuli used was the

same. This was expected, as THP-1 cells express low levels of *CD14* (Bosshart & Heinzelmann, 2004), a membrane-associated protein that forms part of the LPS-receptor complex with TLR4 (Park et al., 2009). This means that THP-1 cells are far less sensitive to LPS stimulation than U937 cells potentially making them a worse model for investigating LRP1 shedding.

However, when I western blotted the conditioned media from LPS-stimulated cells, I could only detect LRP1 in the THP-1 samples after 24 h of stimulation. The lack of detectable LRP1 in the conditioned media of LPS-stimulated U937 cells led me to conclude that the U937 cell line was not an appropriate model for investigating LRP1 shedding from human macrophages. Therefore, despite their insensitivity to LPS, I continued to test THP-1 cells as a potential model.

I increased the number of cells to increase the amount of protein shed and was then able to detect shed LRP1 in the conditioned media of both the unstimulated and LPS-stimulated THP-1 cells at 6 h. Previous work showed that in murine macrophages LPS stimulates rapid shedding of LRP1 within 30 min (Gorovoy et al., 2010), and in primary human macrophages this process was detected after 8 h (Schubert et al., 2019). However, I did not observe any LPS-stimulated shedding at the 6 h time point I measured. This again could be due to THP-1 cells not responding well to LPS.

I therefore investigated whether other inflammatory cytokines that act via different receptors, not requiring CD14, could stimulate LRP1 shedding in THP-1 as reported previously in other cell types (Gorovoy et al., 2010). However, none of the other pro-inflammatory cytokines I tested significantly changed LRP1 shedding at any of the time points I measured.

The lack of any significant change in LRP1 shedding in response to various pro-inflammatory stimuli led me to conclude that PMA-differentiated THP-1 cells were a poor model for studying LRP1 shedding.

Therefore, I decided that both cell lines I tested were not appropriate model systems for my investigation into LRP1 shedding and so I switched my focus to primary human macrophages isolated from peripheral blood.

Chapter 4: Investigating LPS-stimulated LRP1 shedding from primary human macrophages

4.1 Introduction

After demonstrating that neither of the human monocytic cell lines tested (i.e. THP-1 or U937) were appropriate model systems for the investigation of LPS-stimulated LRP1 shedding in macrophages, I decided to investigate primary human monocytes isolated from peripheral blood leukocyte cones which could then be differentiated to macrophages.

These cells are a much more biologically relevant way to investigate monocyte and macrophage function in vitro. Primary macrophages respond better to stimuli and polarisation signals (Shiratori et al., 2017; Spiller et al., 2016) than THP-1 cells, which are considered to be a simplified model of human macrophages in comparison (Tedesco et al., 2018).

Using primary human monocyte-derived macrophages does have some downsides, which is why I started investigations with the cell line. Each leukocyte cone was obtained from a different, anonymous donor, which means that there is a large amount of variation that cannot be controlled for, not only in the genetic background of the donor, but also the inflammatory status of the donor at the time when the blood was donated. These primary cells also don't proliferate and cannot be stored in liquid nitrogen, so the number of cells isolated from each donor is finite and there is little opportunity to conduct multiple experiments on each donor. This made working with primary human monocyte-derived macrophages a more expensive and time-consuming process then relying on cell lines.

However, as I have discussed in the previous chapter, THP-1 and U937 cells were not appropriate models for investigating the LPS-stimulated shedding of LRP1. Therefore, despite the downsides, I wanted to investigate whether primary human monocyte-derived macrophages would be a better model of this process. Macrophages differentiated from peripheral blood monocytes have much higher levels of *CD14* expression than THP-1 cells, making them much more sensitive to LPS stimulation (Bosshart & Heinzelmann, 2004).

4.2 Primary human macrophage polarisation

In order to confirm that the cells that I generated from leukocyte cones were monocyte-derived macrophages, I used combinations of cytokines to differentiate the isolated monocytes to macrophages, and then to polarise them to either pro- or anti-inflammatory phenotypes. I then used qRT-PCR to measure the expression of various genes which are markers of polarisation, to validate that the cell phenotype was as expected.

4.2.1 Validation of marker expression

I first compared two different protocols for differentiating macrophages from the primary human monocytes. The monocytes were either cultured with M-CSF (100 ng/mL), or media conditioned by L929 cells (30%), for 7 days. L929 cells are an immortalised murine fibroblast cell line which secrete high amounts of M-CSF and the conditioned media from these cells has been used as an alternative to recombinant M-CSF for differentiation of monocytes into macrophages in vitro (de Brito Monteiro et al., 2020; Trouplin et al., 2013). I investigated which method would be most appropriate to produce unpolarised macrophages which could then be polarised into pro- or anti-inflammatory phenotypes.

I counted the number of macrophages after 7 days of treatment with either M-CSF or L929 conditioned media treatment using a haemocytometer and found that M-CSF treatment resulted in a higher number of cells (Figure 4.1A). I used a combination of LPS (100 ng/mL) and IFN-γ (50 ng/mL) for 24 h to polarise the cells to a pro-inflammatory phenotype (Higuchi et al., 2016) and the combination of IL-4 (20 ng/mL) and IL-13 (20 ng/mL) for 24 h to produce an anti-inflammatory phenotype (Rogers et al., 2019). I then measured the expression of *CD14* as a monocytic marker, *CD80* as a pro-inflammatory marker, and *CD206* as an anti-inflammatory marker (Ambarus et al., 2012). *CD14* expression was downregulated following polarisation to either macrophage phenotype, and *CD80* and *CD206* were upregulated following polarisation to pro-inflammatory and anti-inflammatory phenotype respectively (Figure 4.1B).

As the two methods produced macrophages which responded to polarisation in a similar fashion, I decided to use recombinant M-CSF for all further macrophage differentiations since this produced the greater number of cells.

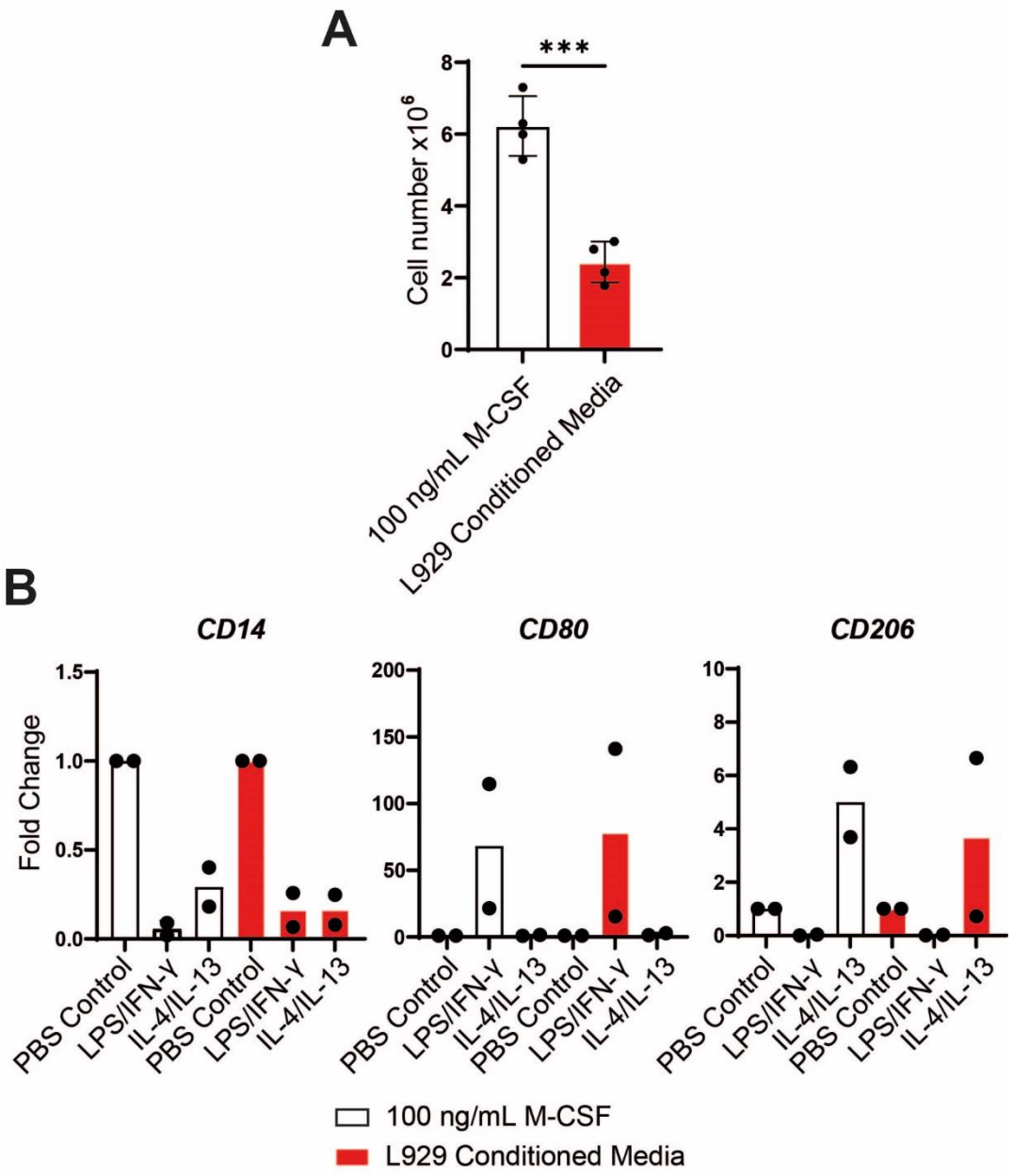

Figure 4.1 Differentiation with M-CSF generated more macrophages than differentiation with conditioned media from L929 cells.

(A) Peripheral blood monocytes ($20x10^6$) were plated in 10 cm cell culture dishes and treated for 7 days with either M-CSF (100 ng/mL) or conditioned media from L929 cells (30%). Cells were dissociated with EDTA (0.02%) and removed from the dishes using a cell lifter. Cell number was counted using a haemocytometer. Data were tested for normality with a Shapiro-Wilk and analysed using an unpaired t-test (mean ± SD, 4 independent donors, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (B) Primary human macrophages after 7 days of treatment with either M-CSF (100 ng/mL) or conditioned media from L929 cells (30%) were treated with LPS (100 ng/mL) and IFN-γ (50 ng/mL), IL-4 (20 ng/mL) and IL-13 (20 ng/mL), or PBS as a control for 24 h. RNA was isolated and reverse transcribed to cDNA. Relative gene expression was calculated by qRT-PCR relative to the housekeeper *GAPDH* and fold change was compared relative to PBS-treated cells (mean, 2 independent donors).

To further validate my protocol for polarising M-CSF-differentiated human macrophages, I quantified the expression of additional markers associated with polarisation to pro- or anti-inflammatory phenotypes in three additional donors (Figure 4.2) (Ambarus et al., 2012; Chanput et al., 2014; Vogel et al., 2014). Along with *CD14,* I also measured the expression of *CD16* and *CD68* as general monocyte markers. I measured *IL1β* as another marker of a pro-inflammatory phenotype, and *CD209* as a marker of an anti-inflammatory phenotype. *CD14* and *CD68* were downregulated after 24 h of polarisation into a pro-inflammatory phenotype. In terms of the markers of pro-inflammatory phenotype, *CD80* was strongly upregulated in response to LPS and IFN-γ treatment, however there was more variation in the response of *IL1β* expression meaning that there was not significant upregulation. The expression of both *CD206* and *CD209,* markers of an anti-inflammatory phenotype, was not significantly changed due to the variation in the response between the donors.

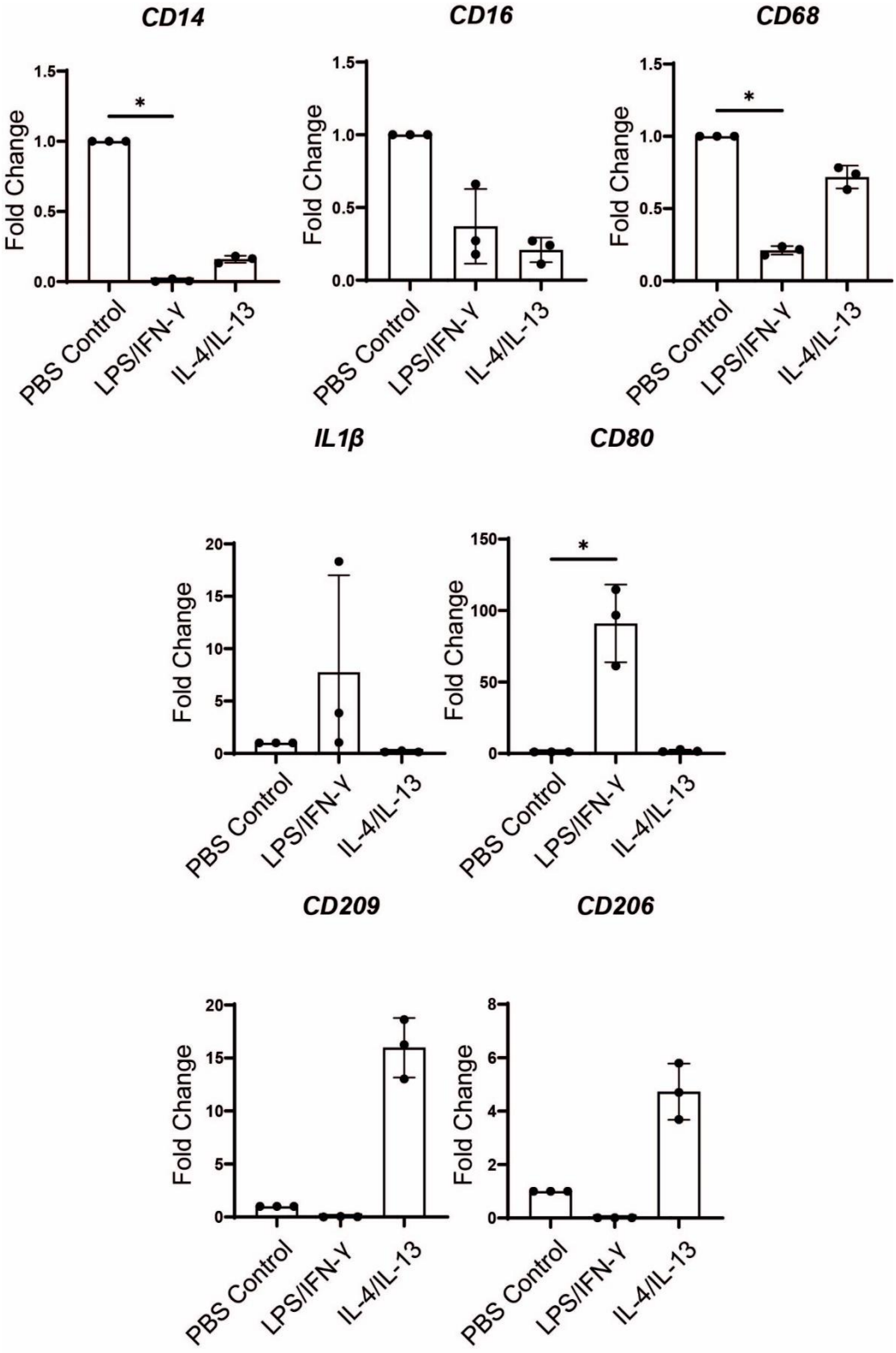

Figure 4.2 Primary human monocyte-derived macrophages were polarised into pro- or anti-inflammatory phenotypes.

Primary human monocyte-derived macrophages were treated with LPS (100 ng/mL) and IFN-γ (50 ng/mL), IL-4 (20 ng/mL) and IL-13 (20 ng/mL), or PBS as a control for 24 h in the presence of serum. RNA was isolated and reverse transcribed to cDNA. Relative gene expression was calculated by qRT-PCR relative to the housekeeper *GAPDH* and fold change was compared relative to PBS-treated cells. Data were analysed using a Kruskal-

Wallis and corrected for multiple comparisons with Dunn's test (mean ± SD, 3 independent donors, * $p < 0.05$).

I planned to investigate how macrophage polarisation affected the shedding of LRP1 using western blotting analysis of the conditioned media. Unfortunately, I found that FBS contains LRP1, (Section 3.2)) which complicated analysis of media from cells grown in serum. I therefore tested whether polarisation of the monocyte-derived macrophages was altered by the absence of serum (Figure 4.3).

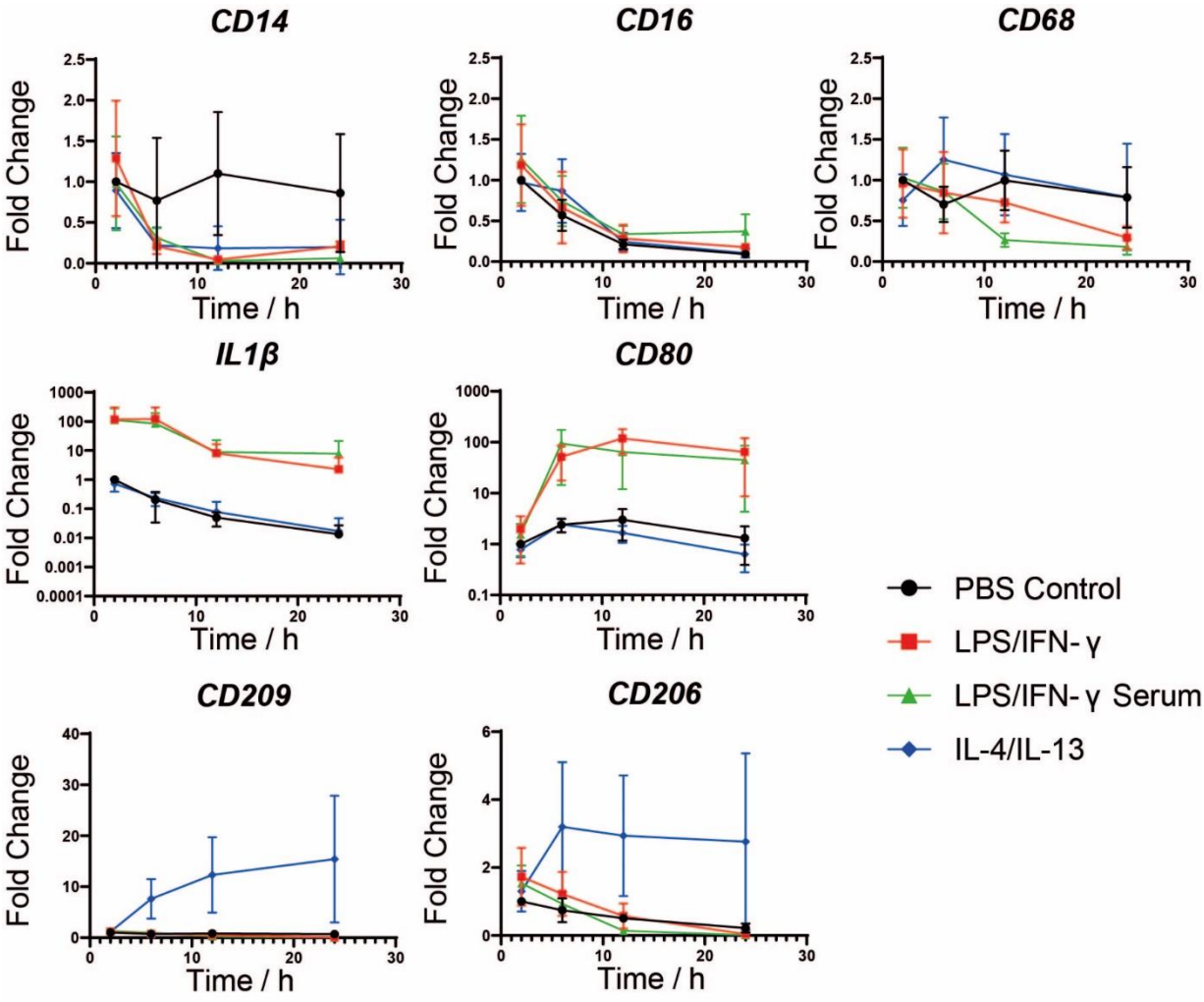

Figure 4.3 Macrophages polarised effectively in the absence of serum.
Primary human macrophages were treated with LPS (100 ng/mL) and IFN-γ (50 ng/mL), IL-4 (20 ng/mL) and IL-13 (20 ng/mL), or PBS as a control for 24 h with or without the presence of serum. RNA was isolated at 2, 6, 12, and 24 h and reverse transcribed to cDNA. Relative gene expression was calculated by qRT-PCR relative to the housekeeper *GAPDH* and fold change was compared relative to PBS-treated cells at 2 h. Expression in polarised cells was compared to that in the PBS-treated condition at each time point using Mann-Whitney U tests (mean ± SD, 3 independent donors).

The variation between donors meant that none of the differences were statistically significant, however the trends are clear and fit with what was observed previously after 24 h of polarisation in serum (Figure 4.2). I found that in the majority of cases, the changes in expression caused by the polarisation happened quickly, within 6 h, and were maintained for the full 24-h time course. This demonstrated that the monocyte-derived macrophages were successfully polarised in the absence of serum.

As another method of evaluating the inflammatory state of the macrophages, I measured TNF release by ELISA (Figure 4.4). I found that 24 h of treatment with LPS and IFN-γ caused a large increase in TNF release compared with the unpolarised cells. This effect was also not seen when cells were polarised to an anti-inflammatory phenotype with IL-4 and IL-13.

Looking at the time course of TNF release, I observed that LPS/IFN-γ treatment caused TNF to be released rapidly (within 2-4 h), with the TNF release plateauing after 12 h. In the presence of serum, LPS and IFN-γ treatment resulted in a quicker release of TNF which plateaued at a higher level than without serum. There was also more variation in the amount of TNF released from the cells in the presence of serum.

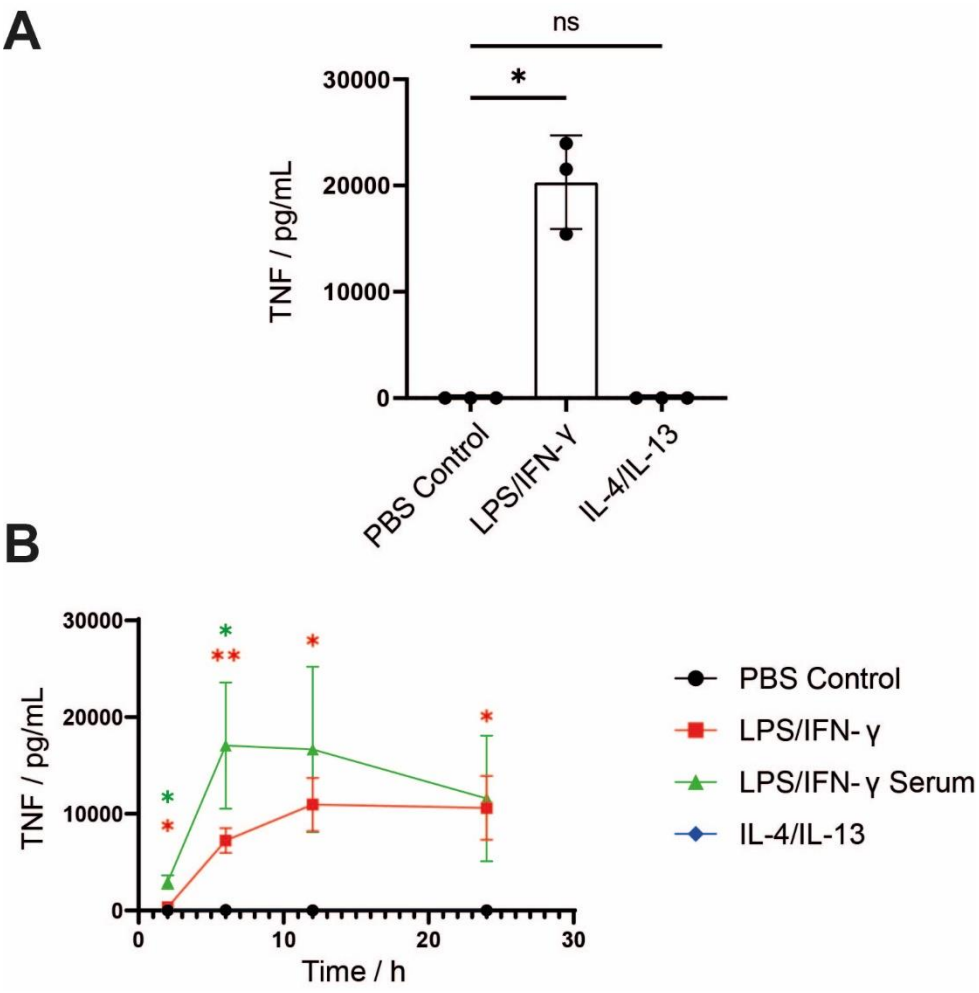

Figure 4.4 Pro-inflammatory polarisation of macrophages resulted in TNF release which plateaued after 12 h.

(A) Primary human macrophages were treated with LPS (100 ng/mL) and IFN-γ (50 ng/mL), IL-4 (20 ng/mL) and IL-13 (20 ng/mL), or PBS as a control for 24 h in the presence of serum. TNF concentration in conditioned media was analysed by ELISA. Data were analysed using a Kruskal-Wallis and corrected for multiple comparisons with Dunn's test (mean ± SD, 3 independent donors, * $p < 0.05$). (B) Primary human macrophages were treated with LPS (100 ng/mL) and IFN-γ (50 ng/mL), IL-4 (20 ng/mL) and IL-13 (20 ng/mL), or PBS as a control for 24 h with or without serum. TNF concentration in conditioned media at 2, 6, 12, and 24 h was analysed by ELISA. Data were tested for normality by Shapiro-Wilk and treated conditions were compared to the untreated condition at each time point using a two-way ANOVA and corrected for multiple comparisons with Dunnett's test (mean ± SD, 4 independent donors, * $p < 0.05$, ** $p < 0.01$).

Following on from the results of the polarisation experiments I concluded that I was able to successfully isolate, differentiate, and polarise primary human macrophages to both pro- and anti-inflammatory phenotypes, and that the cells responded to polarising treatments regardless of the presence of serum in the growth media. Therefore, I could continue with

the western blot analysis of LRP1 shedding from these cells knowing that the lack of serum wasn't greatly affecting how the cells were polarised.

4.2.2 Profiling the expression of genes of interest in M-CSF-derived unpolarised primary macrophages

In order to further profile the primary human macrophages, I measured the basal levels of expression of a number of genes of interest. I looked at the expression of *LRP1, TIMP3,* and *ADAM17,* which are all proteins with key roles in the pathway I am investigating in this research. I then also measured the expression profile of the other active *ADAM*s (Klein & Bischoff, 2011).

I found that the expression of *ADAM20, 30,* and *33,* was not detectable and *ADAM12* and *ADAM19* were not highly expressed in this cell type (Figure 4.5). The rest of the *ADAMs*, *LRP1,* and *TIMP3,* all had a similarly high level of expression in the M-CSF derived, unpolarised macrophages.

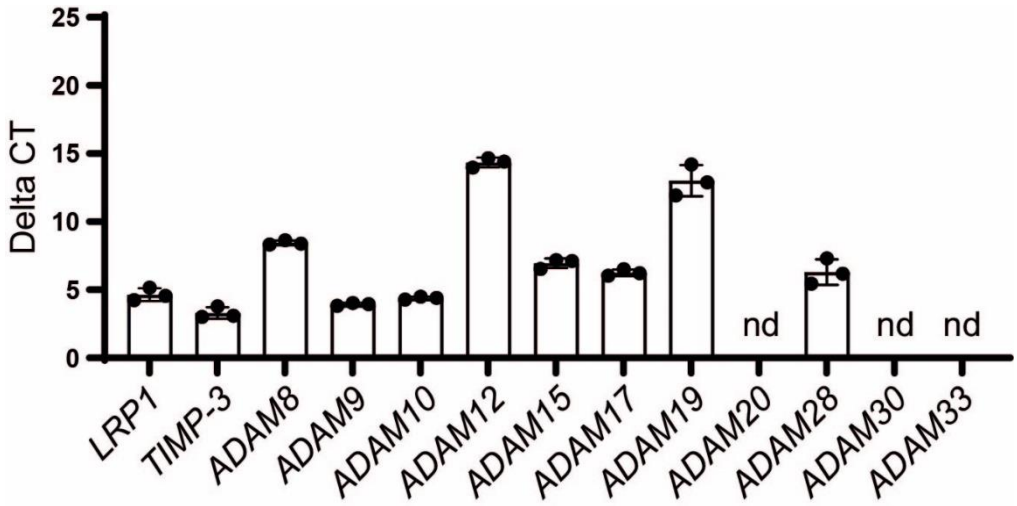

Figure 4.5 *LPR1*, *TIMP3*, and the active *ADAM*s were detected at varying levels of expression in primary human macrophages.
Primary monocyte-derived human macrophages were cultured for 24 h in RPMI 1640 with 10% FBS. RNA was isolated and reverse transcribed to cDNA. Delta CT values of genes of interest were calculated by qRT-PCR (mean ± SD, 3 independent donors).

After profiling levels of expression in unpolarised macrophages, I investigated how 24 h of polarisation changed the expression of these genes (Figure 4.6). I found that pro-inflammatory polarisation caused a significant downregulation in the expression of *LRP1,* while anti-inflammatory polarisation had no significant effect. The expression of *ADAM17* remained stable regardless of how the macrophages were polarised.

There was also no significant change in expression of *ADAM8* or *ADAM12* with polarisation, but *ADAM10* was significantly downregulated by pro-inflammatory polarisation. The expression of *ADAM19* was strongly upregulated (fold change of around 800) by LPS/IFN-γ, but it was expressed at low levels in unpolarised macrophages, so this increase led to expression at a similar level to that of the other ADAMs (Delta CT value of around 23). *ADAM28* was the only one of the genes investigated whose expression was significantly reduced by IL-4/IL-13 anti-inflammatory polarisation.

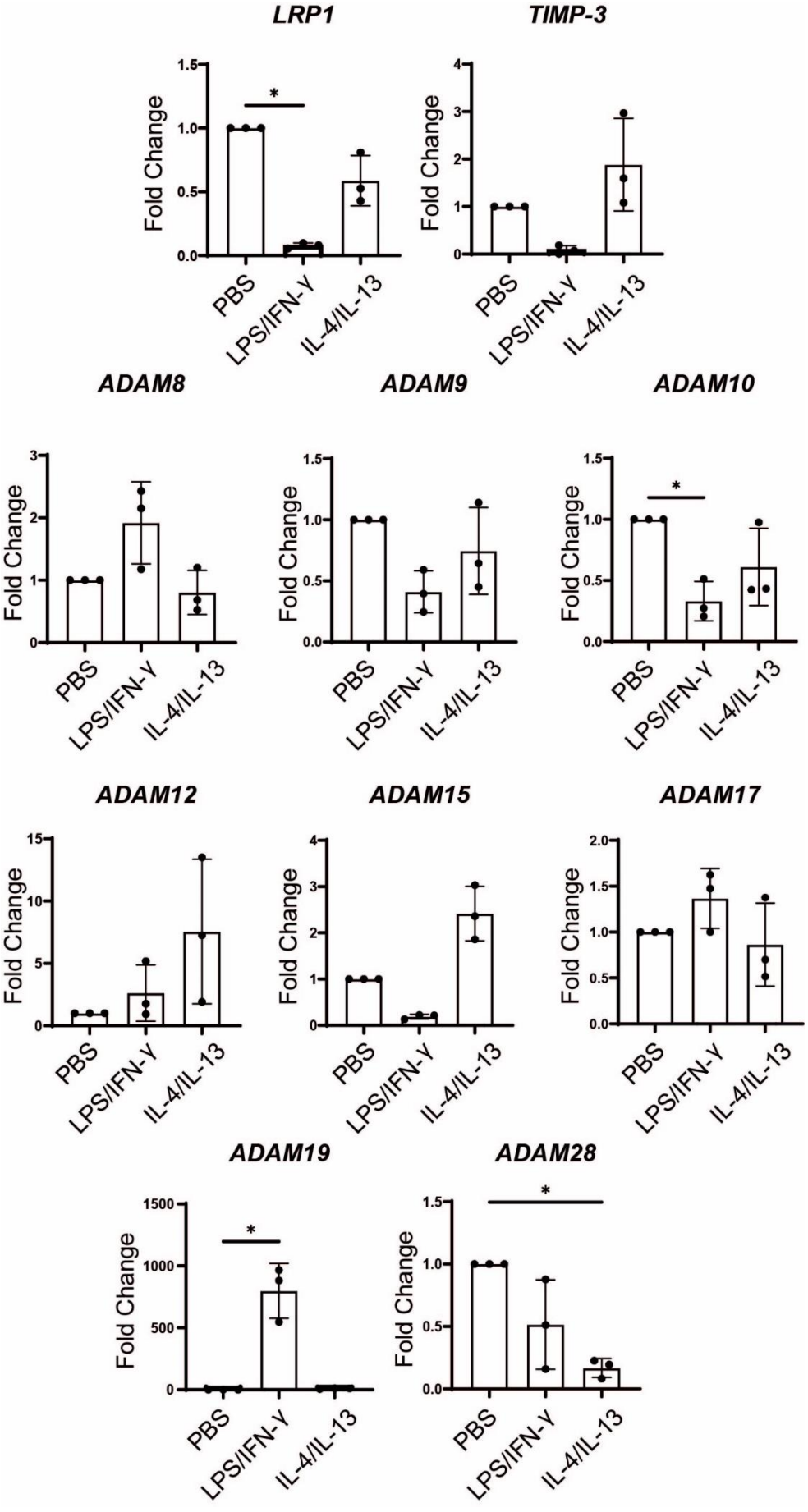

Figure 4.6 Pro-inflammatory polarisation reduced the expression of *LRP1, TIMP3,* and *ADAM10*.

Primary human macrophages were treated with LPS (100 ng/mL) and IFN-γ (50 ng/mL), IL-4 (20 ng/mL) and IL-13 (20 ng/mL), or PBS as a control for 24 h in the presence of serum. Relative gene expression was calculated by qRT-PCR relative to the housekeeper *GAPDH* and fold change was calculated relative to PBS-treated cells. Data were analysed using a Kruskal-Wallis and corrected for multiple comparisons with Dunn's test (mean ± SD, 3 independent donors, * $p < 0.05$).

I then focused on four of these genes, i.e. *LRP1, TIMP3, ADAM17,* and *ADAM10,* and investigated how their expression changed over time during polarisation (Figure 4.7). I included ADAM10 in this experiment as it is the most similar metalloprotease to ADAM17 (Edwards et al., 2009) and so if ADAM17 is not responsible for LRP1 shedding as previously hypothesised (Schubert et al., 2019) then ADAM10 could be an alternative 'sheddase'. As I observed previously (Figure 4.3), there was too much variation between donors for differences to be significant, but some trends are evident.

The reduced expression of *LRP1* and *TIMP3* that was observed at 24 h in response to polarisation with LPS and IFN-γ was only detectable at the 24 h time point. However, the reduced *ADAM10* expression in response to pro-inflammatory polarisation was detectable by 6 h. The expression of *ADAM17* did not change over 24 h regardless of whether the cells were polarised or not. These data, combined with the previous experiment (Figure 4.3) further show that the absence of serum in the growth media during polarisation did not affect how the cells responded to polarising stimuli.

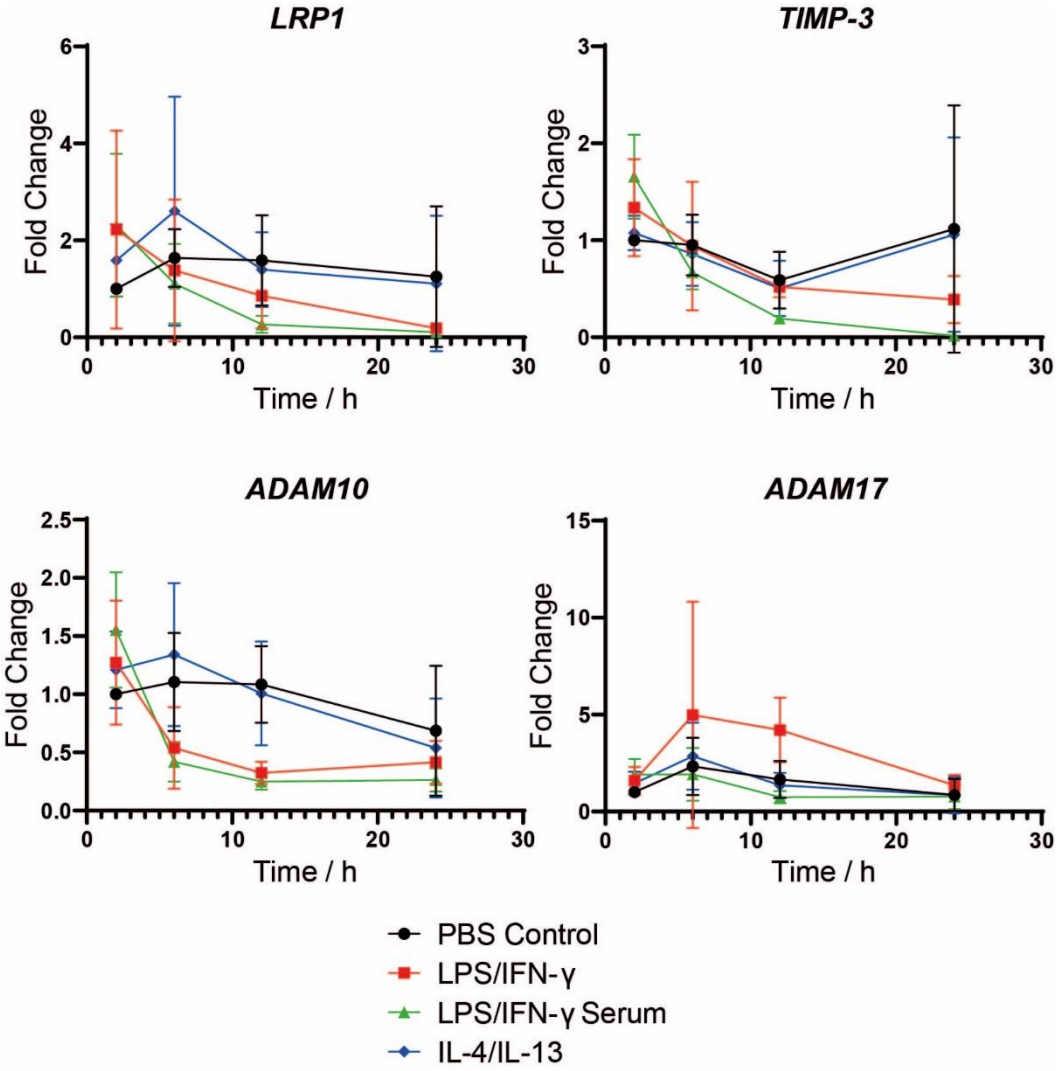

Figure 4.7 Macrophages polarisation altered the expression of *LRP1, TIMP3,* and ADAM10 over time irrespective of the presence of serum.

Primary human macrophages were treated with LPS (100 ng/mL) and IFN-γ (50 ng/mL), IL-4 (20 ng/mL) and IL-13 (20 ng/mL), or PBS as a control for 24 h with or without serum as indicated. RNA was isolated at 2, 6, 12, and 24 h and reverse transcribed to cDNA. Relative gene expression was calculated by qRT-PCR relative to the housekeeper *GAPDH* and fold change was compared relative to untreated cells at 2 h. Treated conditions were compared to the untreated condition at each time point using multiple Mann-Whitney U tests (mean ± SD, 3 independent donors).

4.2.3 LRP1 shedding during polarisation

I immunoblotted the conditioned media from primary macrophages which had been polarised as previously described (Section 4.2.1) for LRP1 (Figure 4.8). I found that over 24 h, the unpolarised cells shed LRP1 into the conditioned media at a consistent rate. The shedding of LRP1 was not significantly affected by pro-inflammatory polarisation with LPS

and IFN-γ at any time point. Macrophages polarised with IL-4 and IL-13 did not significantly change LRP1 shedding.

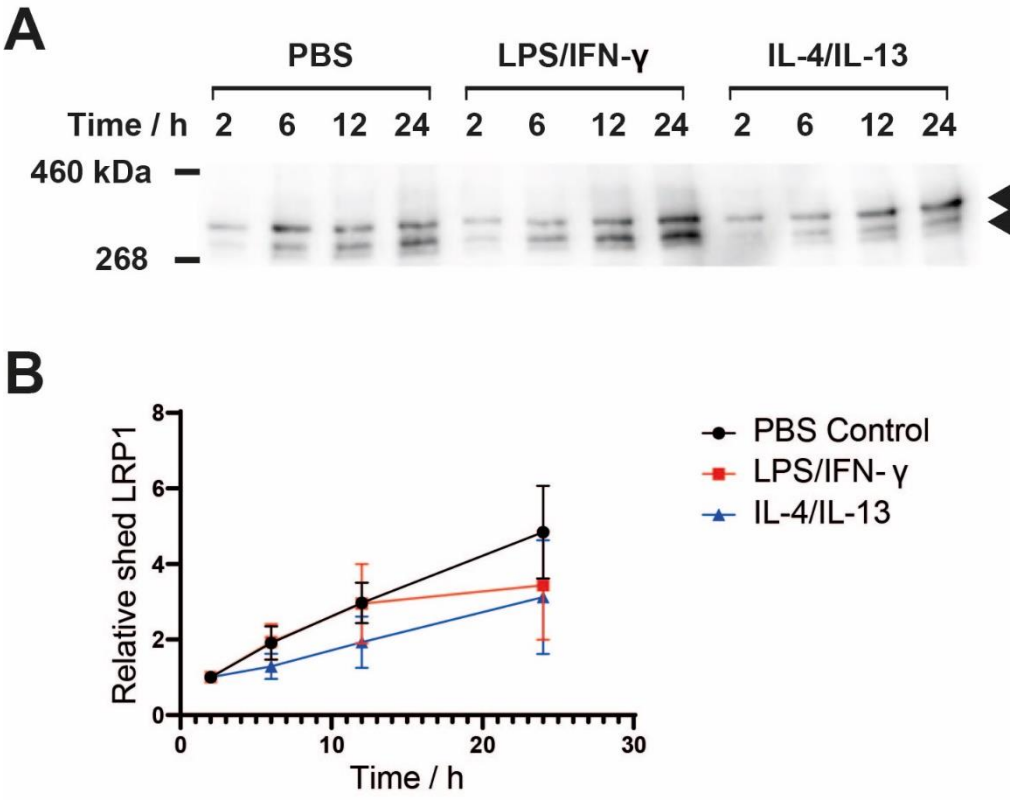

Figure 4.8 Polarisation had no effect on the shedding of LRP1 over 24 h.
Primary human macrophages were treated with LPS (100 ng/mL) and IFN-γ (50 ng/mL), IL-4 (20 ng/mL) and IL-13 (20 ng/mL), or PBS as a control for 24 h without the presence of serum. Conditioned media were collected at 2, 6, 12, and 24 h and concentrated with TCA before analysis by SDS-PAGE and immunoblotting for LRP1 (A). Bands (◀) were quantified and normalised against a Revert Total Protein Stain and shed LRP1 levels calculated relative to the untreated cells at 2 h (B). Treated conditions were compared to the untreated condition at each time point using multiple Mann-Whitney U tests (mean ± SD, 3 independent donors).

4.3 Effect of LPS on LRP1 shedding by primary macrophages

It has previously been observed that LRP1 shedding from a murine macrophage cell line was induced by LPS stimulation and also by IFN-γ stimulation (Gorovoy et al., 2010). It has also been shown that LPS stimulation can induce LRP1 shedding from primary human macrophages (Schubert et al., 2019) however this was shown using flow cytometry and so I wanted to test if I could detect this in primary human macrophages by western blotting.

4.3.1 LPS stimulated the shedding of LRP1

I stimulated primary human macrophages with LPS (100 ng/mL) for 24 h and collected media at 2, 6, 12, and 24 h. I first measured LPS-stimulated TNF release over the 24 h time course (Figure 4.9), and found that TNF release followed the same pattern as I observed previously after stimulation with both LPS and IFN-γ together (Figure 4.4). However, the plateau of TNF release reached by 12 h of LPS stimulation was lower than when the cells were stimulated with LPS in combination with IFN-γ.

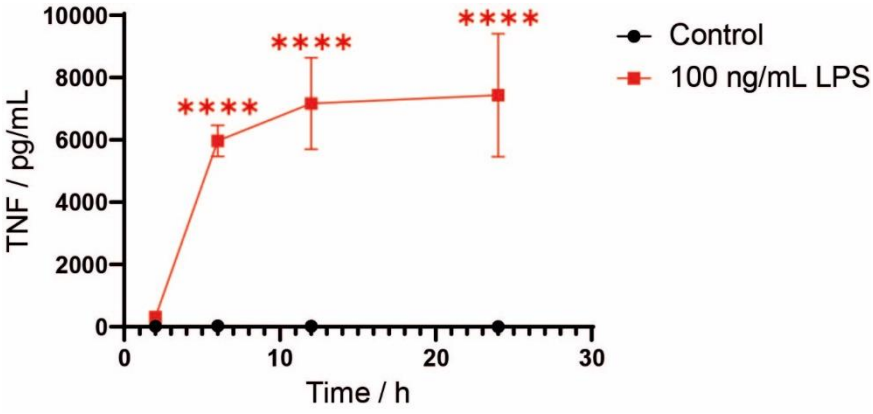

Figure 4.9 LPS stimulation caused a significant TNF release that plateaued after 12 h.

Primary human macrophages were stimulated with LPS (100 ng/mL) or control serum-free media for 24 h. TNF in conditioned media at 2, 6, 12, and 24 h was quantified by ELISA. Data were tested for normality by Shapiro-Wilk and LPS-stimulated conditions were compared to the unstimulated conditions at each time point using multiple Mann-Whitney U tests (mean ± SD, 4 independent donors, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

I then measured the levels of shed LRP1 in the conditioned media. As observed previously, there was a lot of variation between donors in the response to LPS stimulation. This meant that there was no significant difference in LRP1 shedding when the cells were stimulated with LPS (Figure 4.10A and B). However, as there was a small increase in shed LRP1 levels at 6 h, I decided to investigate this time point further with more donors (Figure 4.10C and D). With a larger sample size, I found that 6 h of LPS stimulation did cause a significant increase in the levels of shed LRP1 which is consistent with the literature (Gorovoy et al., 2010; Schubert et al., 2019). However, it is clear from these data that the level to which LRP1 shedding is stimulated by LPS is very variable between donors. Despite this variation, I was able to conclude that I could detect LPS-stimulated LRP1 shedding from primary human macrophages via western blotting of the conditioned media.

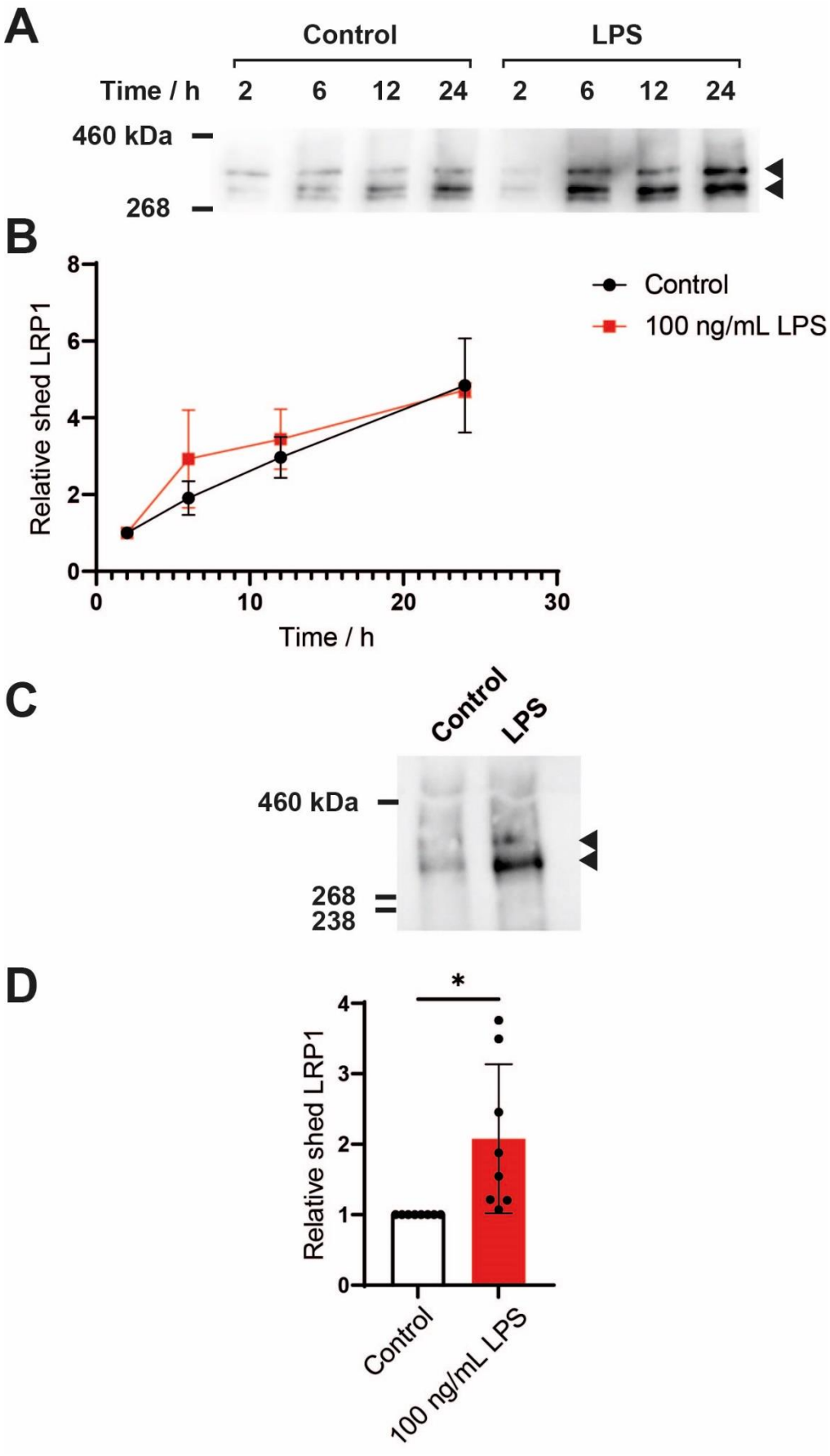

Figure 4.10 LPS stimulation increased shedding of LRP1.

(A) Primary human macrophages were stimulated with LPS (100 ng/mL) or control serum-free media for 24 h. Conditioned media were collected at 2, 6, 12, and 24 h, concentrated

with TCA and analysed by SDS-PAGE and immunoblotting for LRP1. Bands (◄) were quantified and normalised against a Revert Total Protein Stain and shed LRP1 levels calculated relative to the unstimulated control at 2 h (B). LPS-stimulated conditions were compared to the unstimulated conditions at each time point using a two-way ANOVA and corrected for multiple comparisons with Šídák's test (mean ± SD, 3 independent donors). (C) Primary human macrophages were stimulated with LPS (100 ng/mL) or control media for 6 h. Conditioned media were collected and concentrated with TCA before analysis by SDS-PAGE and immunoblotting for LRP1. Bands (◄) were quantified and normalised against a Revert Total Protein Stain and shed LRP1 levels calculated relative to the unstimulated control (D). Data were analysed using a Wilcoxon test (mean ± SD, 8 independent donors, * $p < 0.05$).

4.3.2 Investigating the effects of different concentrations of LPS-stimulation

In order to optimise consistent detection of LPS-stimulated LRP1 shedding, I investigated whether the concentration of LPS used might have an impact. Up to this point I had used 100 ng/mL of LPS to stimulate cells, as this was the concentration shown to stimulate LRP1 shedding in primary human macrophages (Schubert et al., 2019).

Initially, I tested the effect of a range of concentrations of LPS (0.2 – 2000 ng/mL) on cell viability and TNF release (Figure 4.11). 6 h of LPS stimulation did not impact cell viability at any of the concentrations tested and TNF release did not significantly change in a dose-dependent manner. There was a small decrease in TNF release at the highest concentrations of LPS, but the variation meant that this was not significant.

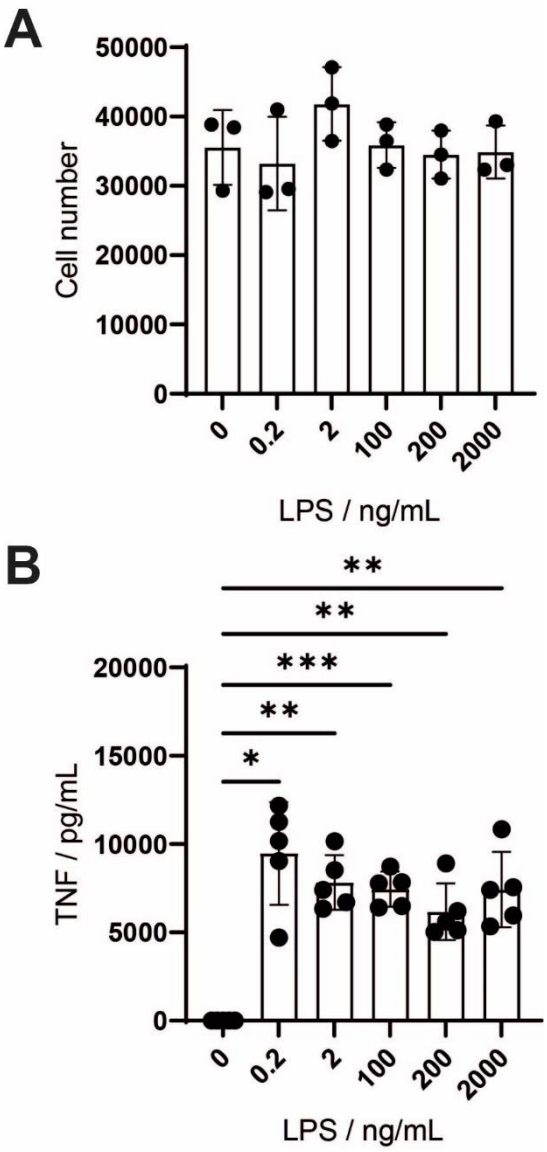

Figure 4.11 All concentration of LPS tested did not affect cell viability and LPS-stimulated TNF release was independent of LPS dose.

Primary human macrophages were stimulated with a range of concentrations (0.2 – 2000 ng/mL) of LPS for 6 h. (A) Cell proliferation was measured using an MTS assay. LPS-stimulated conditions were compared with the unstimulated control using a one-way ANOVA and corrected for multiple comparisons with Dunnett's test (mean ± SD, 3 technical replicates). (B) TNF concentration in conditioned media was analysed by ELISA. Data were tested for normality by Shapiro-Wilk and all conditions were compared using a one-way ANOVA and corrected for multiple comparisons with Tukey's test (mean ± SD, 5 independent donors, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

I then investigated whether LPS dose affected LPS-stimulated shedding of LRP1 (Figure 4.12). I found that at both concentrations of LPS tested, I could detect LPS-stimulated LRP1 shedding after 6 h of stimulation, and that the amount of shed LRP1 was the same at both concentrations. There was also similar variation in LPS-stimulated LRP1 shedding between

donors at each LPS concentration. Considering this in combination with the results shown in the previous figure, I concluded that release of TNF and LRP1 was independent of LPS concentration in the range tested.

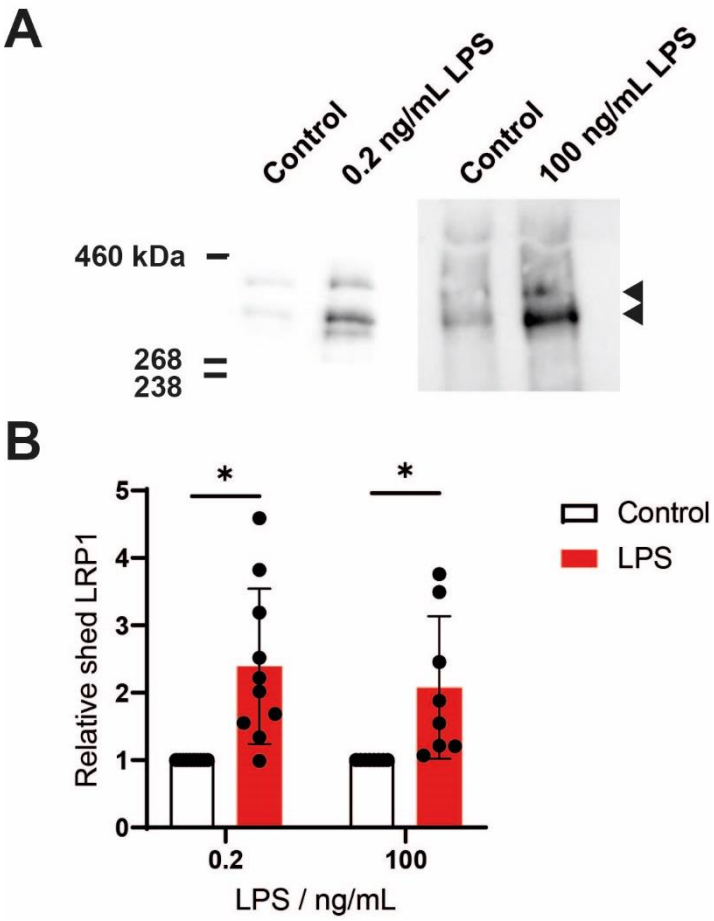

Figure 4.12 LPS-stimulated shedding of LRP1 was comparable at 0.2 and 100 ng/mL of LPS.

Primary human macrophages were stimulated with LPS (0.2 or 100 ng/mL) or PBS (control) for 6 h. Conditioned media were collected and concentrated with TCA before analysis by SDS-PAGE and immunoblotting for LRP1. Bands (◀) were quantified and normalised against a Revert Total Protein Stain and shed LRP1 levels calculated relative to the unstimulated control. Data were analysed using multiple Mann-Whitney U tests (mean ± SD, 10 independent donors for 0.2 ng/mL LPS and 8 independent donors for 100 ng/mL LPS, * $p < 0.05$).

4.4 Discussion

The differentiation of primary human monocytes, isolated from peripheral blood of healthy donors, into macrophages is an established method for studying the biological functions of these cells in vitro, and results in a more physiologically relevant and complex system than the use of macrophage-like cells lines such as THP-1 (Shiratori et al., 2017; Tedesco et al.,

2018). For this reason, it was important to establish a method for the isolation, differentiation, and polarisation of primary human macrophages in order to investigate how the shedding of LRP1 is regulated in this cell type.

Once I had isolated the monocytic cells from the leukocyte cones by two-step density centrifugation, I tested two methods to differentiate the monocytes into macrophages. I compared using recombinant M-CSF (100 ng/mL) with using media that had been conditioned by L929 cells (30% of growth media) for 7 days. It has been shown that murine macrophages derived using these methods show different metabolic profiles, and L929-derived macrophages produce slightly lower levels of pro-inflammatory cytokines (de Brito Monteiro et al., 2020). However, as this work was done with murine macrophages, I wanted to investigate which method might produce the most appropriate model for investigating LRP1 shedding in human cells.

Both methods produced macrophages which polarised very similarly in response to either pro- or anti-inflammatory stimuli. However, L929-conditioned media produced significantly fewer macrophages than M-CSF. This greatly reduced the number of experiments I could do with the cells from one donor, meaning that it was not feasible to continue with this method of macrophage differentiation. Therefore, I used M-CSF to differentiate cells for the remainder of my studies.

To confirm that the cells generated after 7 days of M-CSF treatment were indeed macrophages, I used combinations of cytokines to polarise them into either pro- or anti-inflammatory phenotypes and measured the expression of characteristic polarisation markers (Ambarus et al., 2012).

After 24 h of polarisation, the expression of polarisation markers had changed as expected (Vogel et al., 2014). Polarisation with LPS and IFN-γ resulted in upregulation of pro-inflammatory markers (*IL1β* and *CD80*), while polarisation with IL-4 and IL-13 resulted in the upregulation of the anti-inflammatory markers (*CD206* and *CD209*). There was a large spread in the effect size, reflecting variation in the genetic background and immune status of the donors. I also investigated how expression of the polarisation markers changed over time and found that the changes in gene expression generally occurred quickly (within 6 h) and were then maintained for 24 h.

Given that I planned to analyse LRP1 shedding by western blotting of the conditioned media, I investigated whether it was possible to polarise cells in lower amounts of serum, as high serum levels would interfere with this analysis. I initially investigated reducing the serum concentration to 0.1%, but I found that the serum I used contained LRP1 which produced a far stronger western blot signal than the LRP1 secreted from the cells (Section

3.2). I therefore tested serum-free medium and confirmed that macrophages polarised effectively to both pro- and anti-inflammatory phenotypes in the absence of serum.

I also measured how expression of genes I thought might be involved in LRP1 shedding changed in response to polarisation. The expression of *LRP1* and *TIMP3* was downregulated in response to pro-inflammatory polarisation while *ADAM17* expression was unaffected. All three of these proteins are known to be predominantly controlled post-translationally at the cell membrane (Gorovoy et al., 2010; Scilabra et al., 2013; van der Geer, 2002; Yoda et al., 2013; Zurhove et al., 2008), so these changes in gene expression are likely to be secondary feedback responses. This is further supported by the observation that these changes only occurred late in the polarisation time course, rather than as a rapid response to stimuli.

I also measured TNF release from the primary macrophages as another marker of pro-inflammatory polarisation. As expected, polarisation with LPS and IFN-γ resulted in a rapid, large increase in TNF release which peaked at 6 h and then plateaued. There was no significant difference between TNF release in the presence or absence of serum, although this was potentially due to variation between donors, with a trend towards slightly reduced TNF release in serum-free conditions. The amount of TNF released was far higher than that of the cells with the unpolarised or anti-inflammatory phenotype.

The ADAM demonstrating the largest change in expression upon polarisation was *ADAM19,* which was highly upregulated in response to pro-inflammatory polarisation. ADAM19 is a marker of monocyte differentiation to dendritic cells (Fritsche et al., 2000) and is not generally expressed in macrophages (Klein & Bischoff, 2011). Therefore, this upregulation could indicate that the isolation and differentiation protocol I used did not produce an entirely pure culture of macrophages. However, the scale of this upregulation was mainly due the very low level of expression of *ADAM19* in the unpolarised macrophages, and so even with the large fold change, was still not expressed particularly highly compared with the other ADAMs.

Of the other active ADAMs, ADAM10 is the most similar to ADAM17 (Edwards et al., 2009) and it has a minor role in the release of TNF (Zheng et al., 2004). These closely related ADAMs showed different expression patterns upon polarisation, with *ADAM17* expression not markedly altered by polarisation, and *ADAM10* expression reduced by pro-inflammatory cytokines.

Using primary human macrophages, I was able to detect LRP1 shedding in the conditioned media of unpolarised cells after just 2 h. These primary cells had a much higher basal level of detectable LRP1 shedding than the THP-1 cell line shown in the previous chapter. This made it far easier to investigate any changes in LRP1 shedding by western blotting.

I observed a slight reduction in LRP1 shedding in the IL-4 and IL-13 polarised macrophages. However, this was small, and only significant at one time point. It would be interesting to further investigate how LRP1 shedding changed in an anti-inflammatory phenotype but due to time constraints, this fell outside the scope of my research.

I observed that LPS in combination with IFN-γ had no significant effect on LRP1 shedding over 24 h. Previous studies (Gorovoy et al., 2010; Schubert et al., 2019) have described increased shedding of LRP1 in response to pro-inflammatory stimuli, but these authors used LPS or IFN-γ alone, and did not investigate LPS in combination with IFN-γ. This suggests that IFN-γ may suppress the effects of LPS on LRP1 shedding. IFN-γ, through its stimulation of GSK3 activity, disrupts IL-10-mediated LPS-induced feedback inhibition (Hu & Ivashkiv, 2009) demonstrating how the signalling of these two pro-inflammatory cytokines can interact.

I subsequently investigated the effects of LPS alone on LRP1 shedding and observed the LPS-stimulated shedding described by others. Most notably, Gorovoy *et al*. (2010) showed increased LRP1 shedding 0.5, 1, 3, and 6 h after stimulation of murine macrophages with LPS, and Schubert *et al*. (2019) showed increased LRP1 shedding 4, 8, and 24 h after stimulation of human macrophages with LPS. I observed increased shedding of LRP1 only at 6 h after LPS stimulation. Reasons for this may include species differences [I used human macrophages while Gorovoy *et al*. (2010) used a murine macrophage cell line] and differences in methodology [I used western blotting for the α-chain of LRP1 while Schubert *et al*. (2019) used flow cytometry to show a reduction in the cell surface levels of the LRP1 α-chain].

Skurski *et al.* (2021) demonstrated that LPS-stimulated expression of certain genes in murine macrophages was differentially regulated at low and high doses of LPS. While the study did not look at LRP1 shedding, it did demonstrate that LPS-dependent effects can be concentration-dependent. None of the concentrations I tested affected cell viability and TNF release was also independent of the dose of LPS tested, which was consistent with previous data (Skurski et al., 2021).

I observed the same dose-independent effect when it came to LRP1 shedding. Regardless of the concentration of LPS used, I was able to detect around the same level of LPS-stimulated LRP1 shedding, with the same level of variation. Even though this did not solve the issue of variable responses to LPS stimulation, it did provide further evidence to support the conclusion that LPS significantly stimulates the shedding of LRP1 at this time point.

Therefore, I decided to investigate which protease(s) were responsible for LPS-stimulated LRP1 shedding in primary human macrophages.

Chapter 5: Which proteases are responsible for LRP1 shedding?

5.1 Introduction

The main aim of this research was to investigate how the shedding of LRP1 from human macrophages is regulated and what protease or proteases are responsible for this shedding. In the previous chapter I established that I could detect constitutive and LPS-stimulated LRP1 shedding from primary human macrophages using western blotting. This agrees with previously reported data (Schubert et al., 2019) where flow cytometry was used to show LPS-stimulated LRP1 shedding in this cell type.

In other cell types, several different proteases have been implicated in the shedding of LRP1. In HT1080 cells ADAM12 and MT1-MMP were both shown to shed LRP1 (Selvais et al., 2011) and in murine astrocytes, tPA is reportedly involved (Polavarapu et al., 2007). In MEF cells ADAM10 and ADAM17 both play a role (Liu et al., 2009) and in the murine macrophage cell line, RAW 264.7, ADAM17 was principally responsible (Gorovoy et al., 2010).

Metalloproteases are the most common class of protease responsible for LRP1 shedding and as the mouse macrophages are the most physiologically similar cells to human macrophages out of the ones that have currently been tested, ADAM17 was the primary candidate as the LRP1 sheddase in my investigation.

In this chapter I describe evaluation of a range of broad-spectrum protease inhibitors targeting different classes of proteases (Section 5.2), followed by blocking antibodies and inhibitors targeting specific proteases (Sections 5.3 and 5.4) as inhibitors of LRP1 shedding. I will present data on how the protease responsible could be regulated to control cell surface levels of LRP1 (Section 5.6).

5.2 Screen of broad protease inhibitors

To narrow down the search for the LRP1 sheddase to a particular class of protease, I first investigated the effects of broad-spectrum class-specific protease inhibitors on both constitutive and LPS-stimulated LRP1 shedding.

I tested several metalloprotease inhibitors [TNF protease inhibitor-2 (TAPI-2), CT1746, and GM6001] with different inhibitory activities. In vitro GM6001 is the most potent inhibitor of ADAM17 with a K_i of 1.3 nM whereas both TAPI-2 and CT1746 have K_i values of around 120 nM for ADAM17 (Amour et al., 1998; English et al., 2000; Moss & Rasmussen, 2007). However, none of these inhibitors are specific for ADAM17 and have K_i values for other ADAMs, MMPs, and ADAMTSs in the nanomolar range (Amour et al., 1998; English et al., 2000; Moss & Rasmussen, 2007). I tested these along with a cysteine protease inhibitor

[trans-epoxysuccinyl-l-leucylamido-(4-guanido)-butane (E-64)]; a serine protease inhibitor [4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF)]; and an aspartyl protease inhibitor (pepstatin A).

5.2.1 Effect of protease inhibitors on cell viability and TNF release

I measured the effect of the protease inhibitors on cell viability using the MTS assay (Figure 5.1). The macrophages were treated with the inhibitors for 6 h at the concentrations shown. Of the inhibitors which were reconstituted in PBS (AEBSF, E-64, TAPI-2, and pepstatin A) only AEBSF significantly reduced cell viability. Treatment with the DMSO control did not significantly reduce cell number compared to the PBS control. The metalloprotease inhibitors CT1746 and GM6001, which were reconstituted in DMSO, also did not significantly reduce the cell number. This shows that these inhibitors don't significantly impact cell viability.

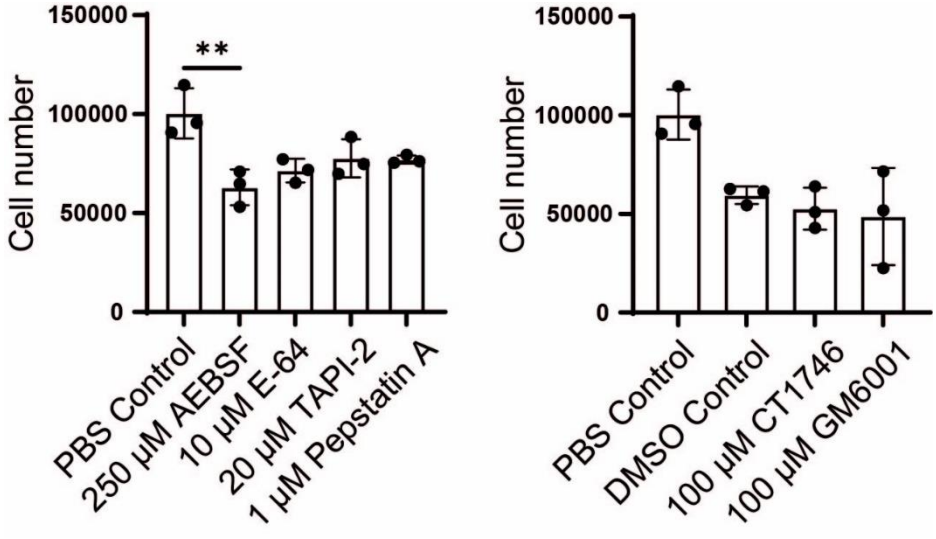

Figure 5.1 Effect of protease inhibitors on viability of primary human macrophages. A range of protease inhibitors were added to primary human macrophages for 6 h at the concentrations shown. Cell viability was measured using an MTS assay. Data were analysed using a Kruskal-Wallis and corrected for multiple comparisons with Dunn's test (mean ± SD, 3 technical replicates, * $p < 0.05$, ** $p < 0.01$).

The concentrations of AEBSF, E-64, TAPI-2, and pepstatin A used are at the low end of the levels required to effectively inhibit protease activity, so I decided to continue using these concentrations for further experiments, while keeping the effects on viability in mind when analysing results. AEBSF has previously been used on primary human monocytes at 250 µM (Basavarajappa et al., 2020) to effectively inhibit serine proteases and E-64 has been used at 10 µM in multiple cell types (Bitu et al., 2013; Yamamoto et al., 2017). TAPI-2 at 20 µM has been shown to inhibit ADAM17 activity with only 20% reduction in cell viability,

however this was done in human colorectal cancer cell lines which could be less sensitive to any toxic effects than primary human macrophages (Wang et al., 2016). Pepstatin A is often used at concentrations higher than 1 μM (Bewley et al., 2011), but I decided to continue with this concentration as it is still significantly higher than the in vitro calculated K_i (Marciniszyn et al., 1976) and any higher concentration could lead to an increased effect on cell viability. Both CT1746 and GM6001 did not themselves affect cell viability and so I decided to continue using them at 100 μM, despite this concentration being higher than the 20-25 μM that has been used previously (Chao et al., 2007; Wang et al., 2021; Yamamoto et al., 2017).

I also investigated the effects of these protease inhibitors on metalloprotease-dependent LPS-stimulated TNF release by ELISA. Due to variation between donors in the amount of TNF released upon LPS stimulation (Figure 5.2A), I also calculated the relative TNF release compared to the LPS-stimulated control or the LPS-stimulated DMSO control for each donor (Figure 5.2B and C). TNF release was significantly inhibited by treatment with the metalloprotease inhibitor CT1746. The metalloprotease inhibitor TAPI-2 did not significantly reduce TNF release. E-64 (cysteine protease inhibitor), pepstatin A (aspartic protease inhibitor), and AEBSF (serine protease inhibitor) all had no effect. These data confirm the class specificity of the inhibitors used.

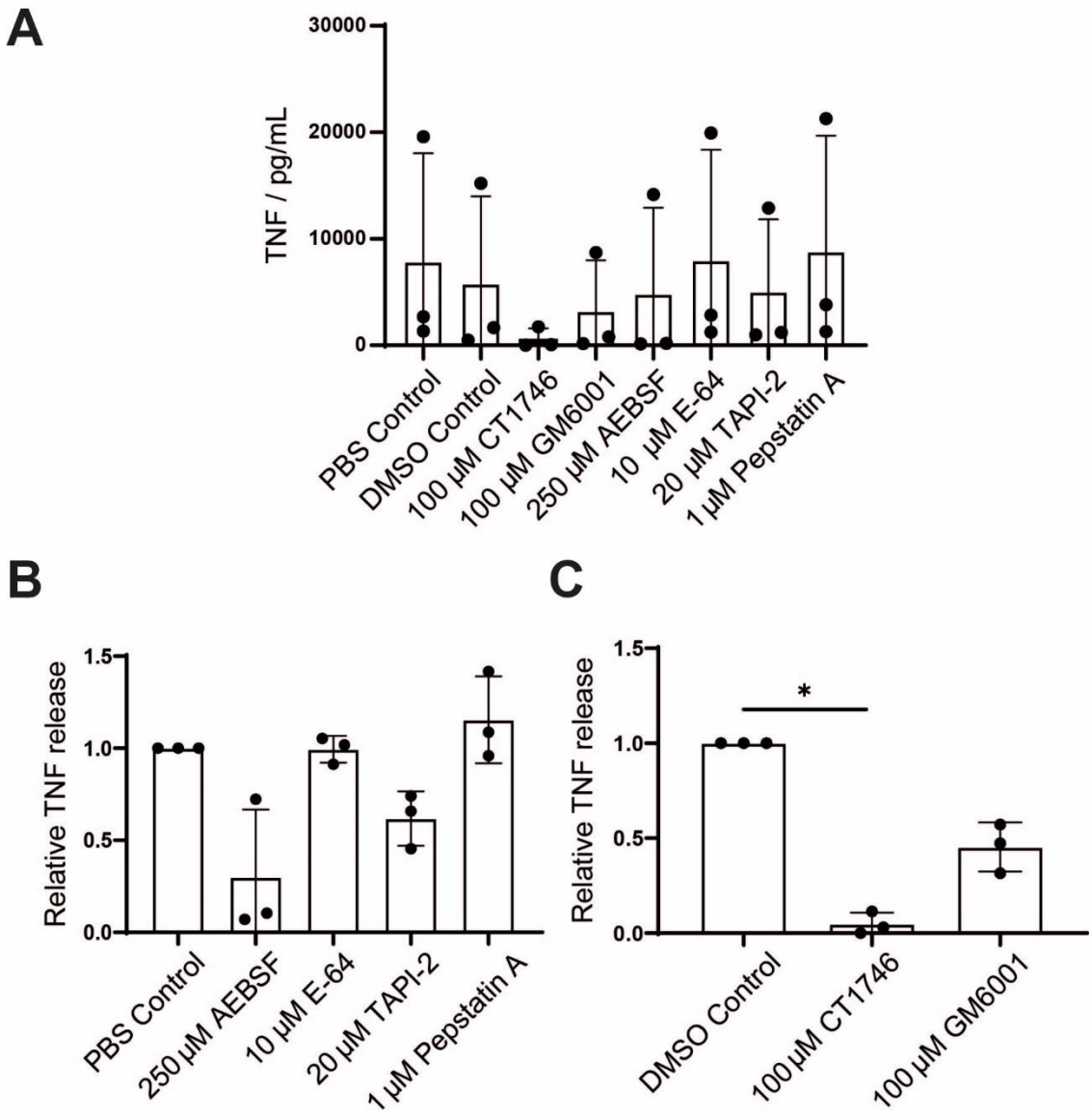

Figure 5.2 Metalloprotease inhibitors inhibited LPS-stimulated TNF release while cysteine and aspartic protease inhibitors did not.

Primary human macrophages were pre-incubated for 1 h with a range of protease inhibitors at the concentrations specified. Cells were stimulated with LPS (0.2 ng/mL) for 6 h in combination with the inhibitors. TNF concentration in conditioned media was analysed by ELISA (A) and then TNF release calculated relative to either the LPS-stimulated control (B) or the LPS-stimulated DMSO control (C). Data were analysed using a Kruskal-Wallis and corrected for multiple comparisons with Dunn's test (mean ± SD, 3 independent donors, * $p < 0.05$).

The effects of the protease inhibitors on TNF release did not correlate with their effects on cell viability. This provided more evidence to support the continued use of these concentrations as I did not observe that the decrease in cell viability affected how the cells responded to LPS.

5.2.2 Effect of protease inhibitors on LRP1 shedding

To investigate which class or classes of protease were responsible for the shedding of LRP1, I tested the effect of these protease inhibitors on LRP1 shedding from primary human macrophages. The constitutive and LPS-stimulated shedding of LRP1 was quantified by western blot of the conditioned media (Figure 5.3). At both concentrations of LPS used, LPS did not significantly stimulate LRP1 shedding due to the variation in the scale of the response observed, except in the DMSO control conditions at 100 ng/mL of LPS. This made it difficult to identify any significant inhibition of LPS-stimulated LRP1 shedding as a result of treatment with the inhibitors.

None of the inhibitors tested significantly reduced either constitutive or LPS-stimulated LRP1 shedding at either of the concentrations of LPS tested. This could be partly explained by the variation in effect size of LPS on LRP1 shedding between donors and so cannot be used to conclusively rule out the involvement of any of these classes of protease.

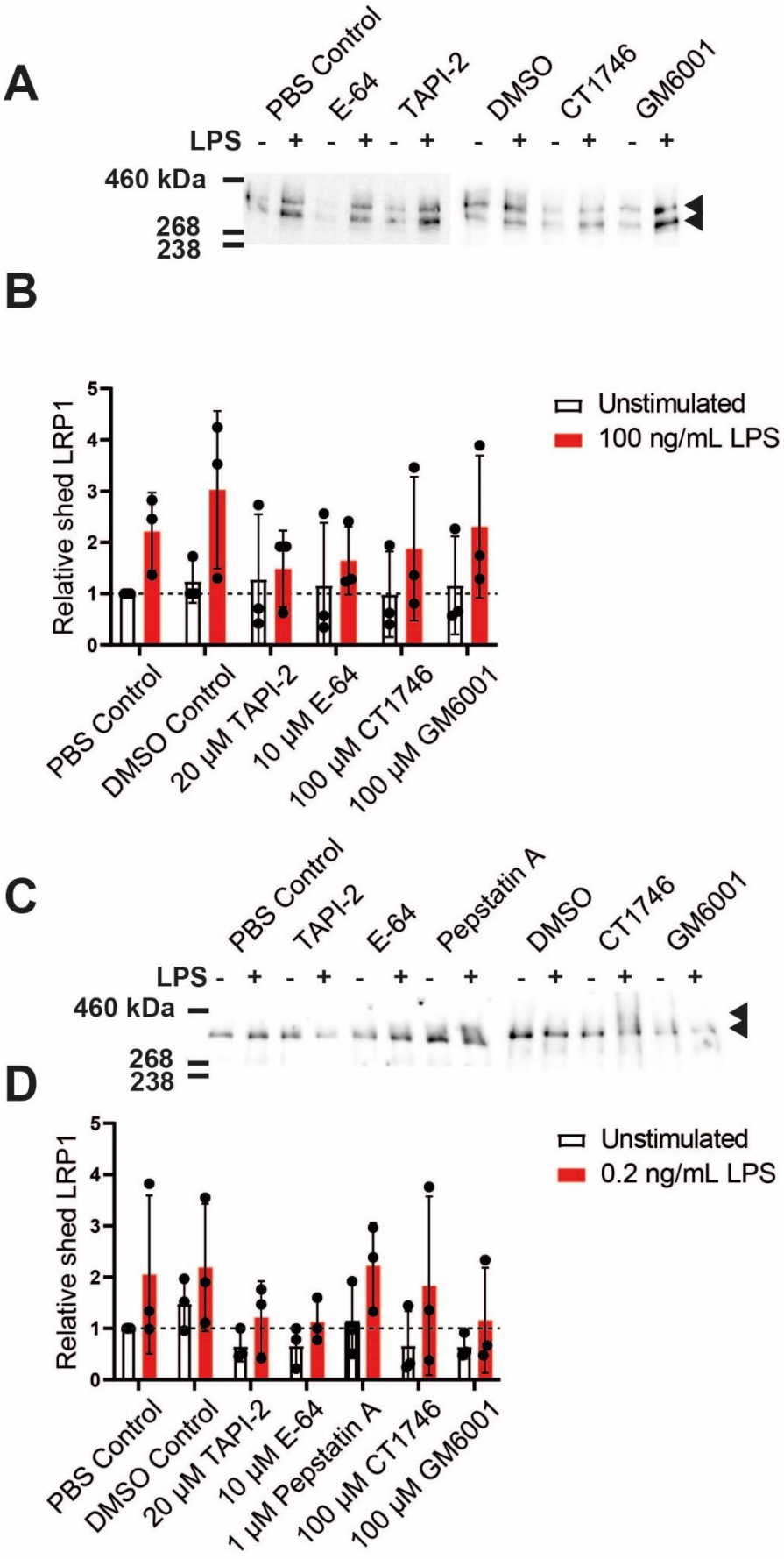

Figure 5.3 Pre-incubation with a range of protease inhibitors did not significantly reduce LPS-stimulated shedding of LRP1.

Primary human macrophages were pre-incubated for 1 h with a range of protease inhibitors at the concentrations specified. Cells were stimulated with LPS at 100 ng/mL (A and B) or 0.2 ng/mL (C and D) for 6 h in combination with the inhibitors. Conditioned media were collected and concentrated with TCA before analysis by SDS-PAGE and immunoblotting for LRP1. Bands (◄) were quantified and normalised against a Revert Total Protein Stain, with shed LRP1 levels calculated relative to the unstimulated control. Unstimulated and LPS-stimulated pairs were compared using multiple Mann-Whitney U tests (mean ± SD, 3 independent donors, * $p < 0.05$).

I also tested the effect of the serine protease inhibitor AEBSF on LRP1 shedding (Figure 5.4). I observed a large increase in levels of LRP1 in the conditioned media when AEBSF-treated macrophages were LPS-stimulated, but this was accompanied by a large increase in the total amount of protein released from the cells. This made it difficult to assess whether AEBSF had any direct effects on LRP1 shedding. Even when the LRP1 band intensities were normalised against the Revert Total Protein Stain, AEBSF treatment increased the levels of shed LRP1.

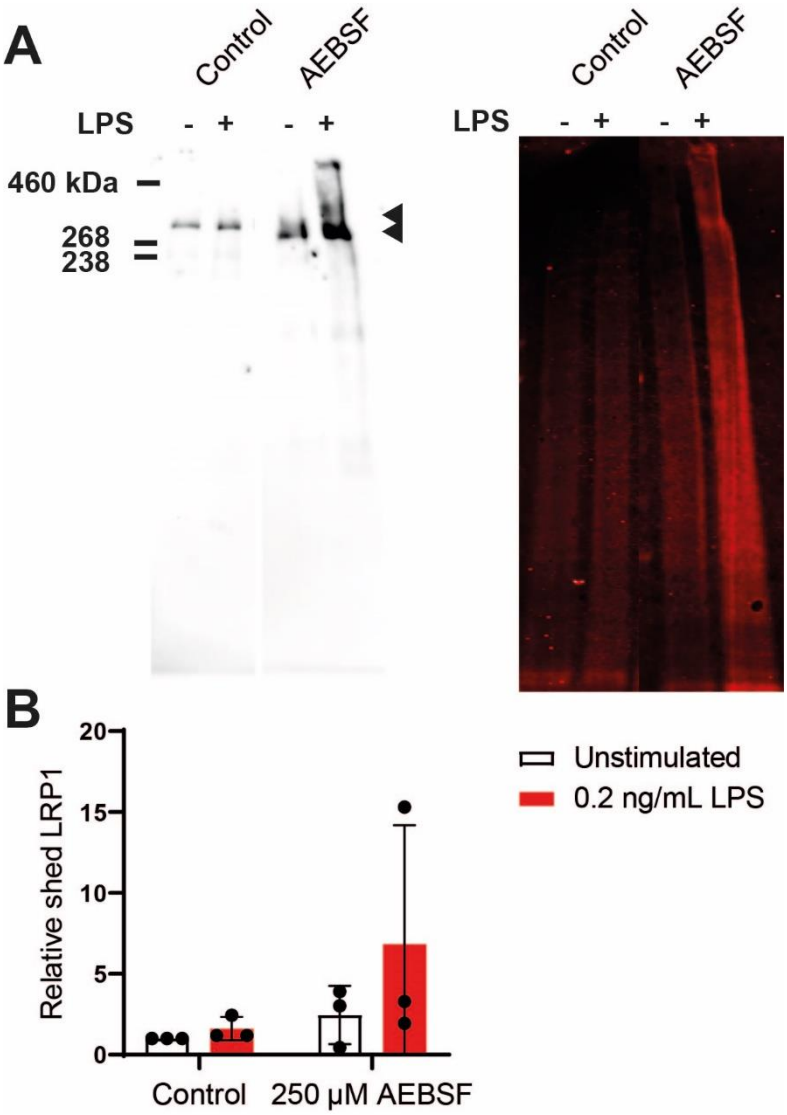

Figure 5.4 AEBSF caused a large increase in total protein release and LRP1 shedding in response to LPS.

Primary human macrophages were pre-incubated for 1 h with AEBSF (250 µM). Cells were stimulated with LPS (0.2 ng/mL) for 6 h in combination with the inhibitor. Conditioned media were collected and concentrated with TCA before analysis by SDS-PAGE and immunoblotting for LRP1 (A). Revert Total Protein Stain of the membrane is also shown to the right of the western blot. Bands (◄) were quantified and normalised against the Revert Total Protein Stain and shed LRP1 levels calculated relative to the unstimulated control (B). Data were analysed using multiple Mann-Whitney U tests (mean ± SD, 3 independent donors).

I therefore tested a different serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), to investigate whether the increased protein release observed with AEBSF was specific to this inhibitor or whether it was a general feature of serine protease inhibition. I only tested this in one donor, so I couldn't perform any statistical tests, but PMSF had no effect on either total protein release or LPS-stimulated LRP1 shedding (Figure 5.5). Taken together

with Figure 5.4, I was able to rule out serine proteases as potential sheddases of LRP1 under LPS-stimulated conditions.

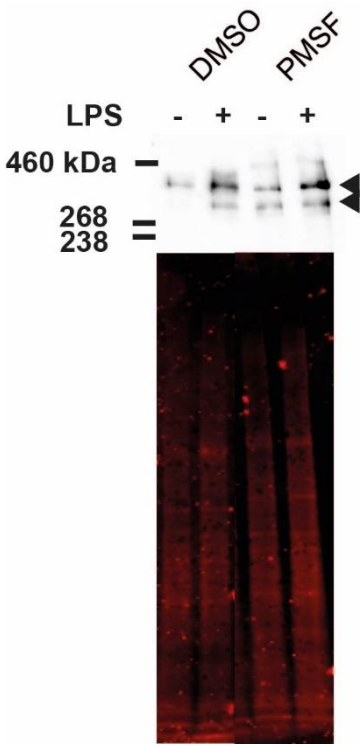

Figure 5.5 The serine protease inhibitor PMSF had no effect on total protein release or LRP1 shedding.

Primary human macrophages were pre-incubated for 1 h with PMSF (1 mM). Cells were stimulated with LPS (0.2 ng/mL) for 6 h in combination with the inhibitor. Conditioned media were collected and concentrated with TCA before analysis by SDS-PAGE and immunoblotting for LRP1. Revert Total Protein Stain of the membrane is also shown below the western blot (n=1).

5.3 Effect of ADAM17 inhibition on LPS-stimulated LRP1 shedding

The screen of broad-spectrum protease inhibitors was inconclusive and so I decided to use more specific methods of inhibition and focus on some likely targets. Due to time limitations, I focused on identifying any responsible metalloproteases. Based on studies in other cell types (Gorovoy et al., 2010; Liu et al., 2009), I considered ADAM17 to be a strong candidate for the LRP1 sheddase and decided to investigate this using more selective methods of ADAM17 targeting.

5.3.1 Inhibition of ADAM17 with the cross-domain antibody D1(A12)

In order to inhibit ADAM17 more specifically, I used the cross-domain human ADAM17 blocking antibody D1(A12) IgG1 (Tape et al., 2011) which has been shown to inhibit cell surface ADAM17 activity significantly more potently than N-TIMP-3 (IC_{50} of 11.2 nM compared to 48.5 nM for TIMP-3).

I treated the macrophages with either D1(A12) or IgG control (both at 0.33 µM) for 6 h and used an MTS assay to assess the effect on cell viability (Figure 5.6A). Neither condition significantly reduced cell number compared to the untreated control.

I analysed conditioned media to investigate the effect of D1(A12) blocking antibody on LPS-stimulated TNF release by ELISA (Figure 5.6B). As expected, I found that the blocking antibody significantly reduced the amount of TNF released in response to 0.2 and 2 ng/mL of LPS. At higher concentrations of LPS, D1(A12) treatment did not significantly reduce TNF release. From this I concluded that 0.33 µM D1(A12) effectively inhibited ADAM17 activity when cells were stimulated with low LPS concentrations without impacting cell viability.

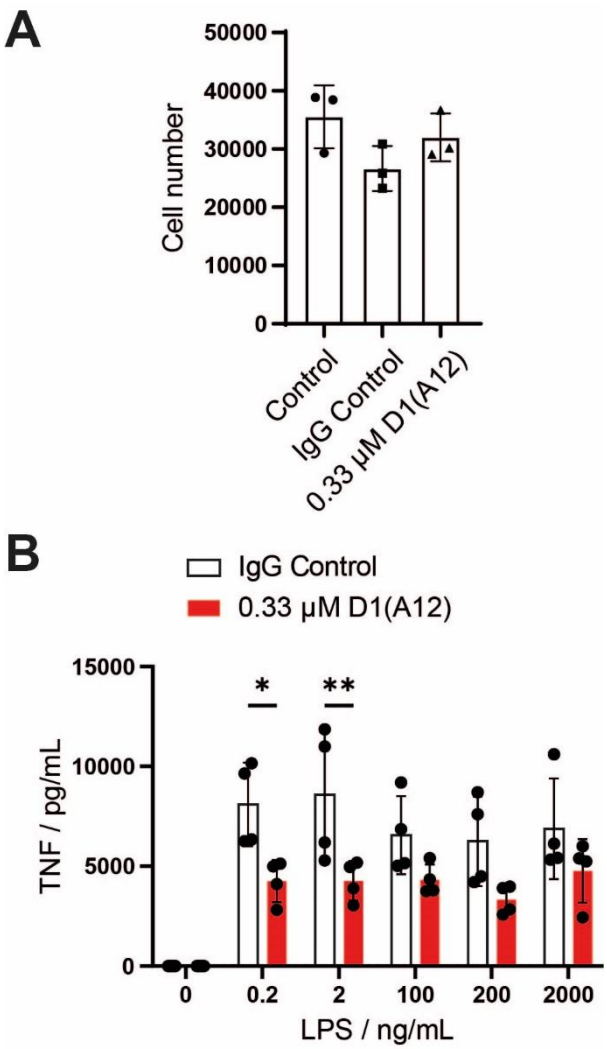

Figure 5.6 Inhibiting ADAM17 with the D1(A12) blocking antibody significantly reduced TNF release in response to low concentrations of LPS, without affecting cell viability.

(A) Primary human macrophages were treated with D1(A12) blocking antibody (0.33 µM) or the same concentration of human IgG for 6 h. Cell number was quantified using an MTS assay. Data were analysed using a one-way ANOVA and corrected for multiple comparisons with Dunnett's test (mean ± SD, 3 technical replicates from 1 donor). (B) Primary human macrophages were pre-incubated for 1 h with D1(A12) blocking antibody (0.33 µM) or the same concentration of human IgG. Cells were stimulated with LPS at a range of concentrations (0.2 – 2000 ng/mL) for 6 h in combination with the blocking antibody. TNF concentration in conditioned media was analysed by ELISA. Data were tested for normality by Shapiro-Wilk and analysed using a two-way ANOVA and corrected for multiple comparisons with Šídák's test (mean ± SD, 4 independent donors, * $p < 0.05$, ** $p < 0.01$).

I then tested the effect of D1(A12) on LPS-stimulated LRP1 shedding and saw that pre-incubation of cells with the D1(A12) blocking antibody had no effect on either constitutive

or LPS-stimulated LRP1 shedding (Figure 5.7) at a range of LPS concentrations. This indicated that ADAM17 was not the metalloprotease responsible for either constitutive or LPS-stimulated LRP1 shedding.

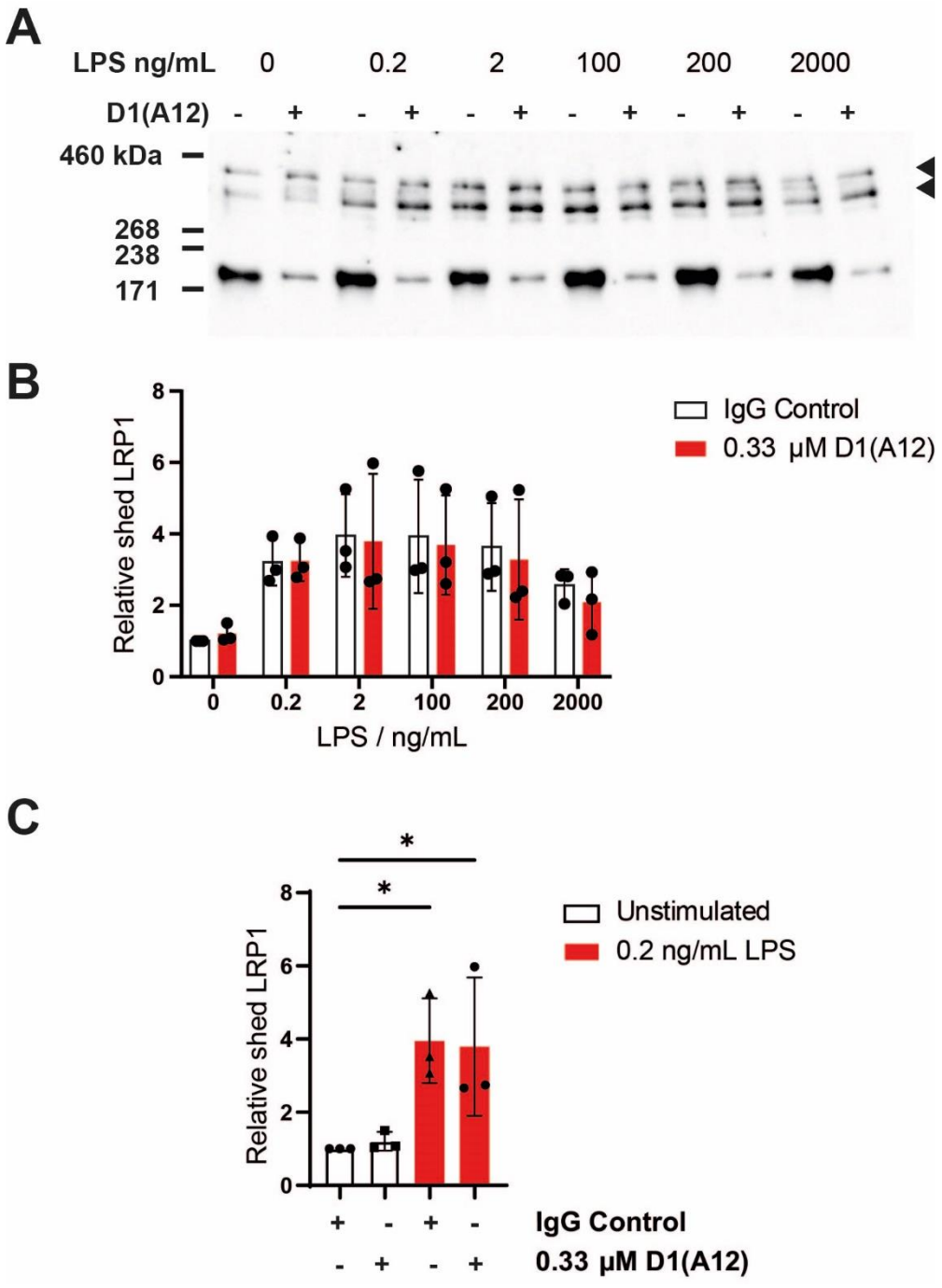

Figure 5.7 Inhibiting ADAM17 with the D1(A12) blocking antibody had no effect on either constitutive or LPS-stimulated shedding of LRP1.

Primary human macrophages were pre-incubated for 1 h with D1(A12) blocking antibody (0.33 µM) or the same concentration of human IgG. Cells were stimulated with LPS at a range of concentrations (0.2 – 2000 ng/mL) for 6 h in combination with the blocking antibody. Conditioned media were collected and concentrated with TCA before analysis by SDS-PAGE and immunoblotting for LRP1 (A). Bands (◀) were quantified and normalised

against a Revert Total Protein Stain and shed LRP1 levels calculated relative to the unstimulated control (B and C). Data were analysed using a Kruskal-Wallis and corrected for multiple comparisons with Dunn's test (mean ± SD, 3 independent donors, * $p < 0.05$).

On the western blots there was a lower molecular weight band (180 kDa) present in both the IgG control and D1(A12) lanes which I did not observe when using any other treatment conditions. Western blot analysis of the antibodies alone confirmed this band to be IgG (data not shown).

As I discussed in the previous chapter, it has been shown that stimulation of murine macrophages with low doses of LPS initiates an ADAM17/iRhom2-dependent feed-forward mechanism that perpetuates inflammation by stimulation of *Tnf and Il1b* mRNA expression (Skurski et al., 2021). Inhibiting ADAM17 was shown to block this enhancement of *Tnf* and *Il1b* expression (Skurski et al., 2021). As I had access to a highly selective ADAM17 inhibitor, I was curious to test whether ADAM17 in human macrophages similarly promotes *TNF* and *IL1β* expression. I used qRT-PCR to quantify the expression of *TNF* and *IL1β* relative to the housekeeper gene *GAPDH* (Figure 5.8), and I found that all concentrations of LPS used stimulated a large increase in the expression of both *TNF* and *IL1β*. The scale of response to LPS of *TNF* expression was very variable between the donors but the trend was consistent. Treatment with the ADAM17 inhibitor had no significant effect on the LPS stimulated increased expression of either gene regardless of concentration of LPS. This adds to previous studies (Mestas & Hughes, 2004; Spiller et al., 2016; Vijayan et al., 2019) which demonstrate another difference between how human and murine macrophages respond to LPS stimulation.

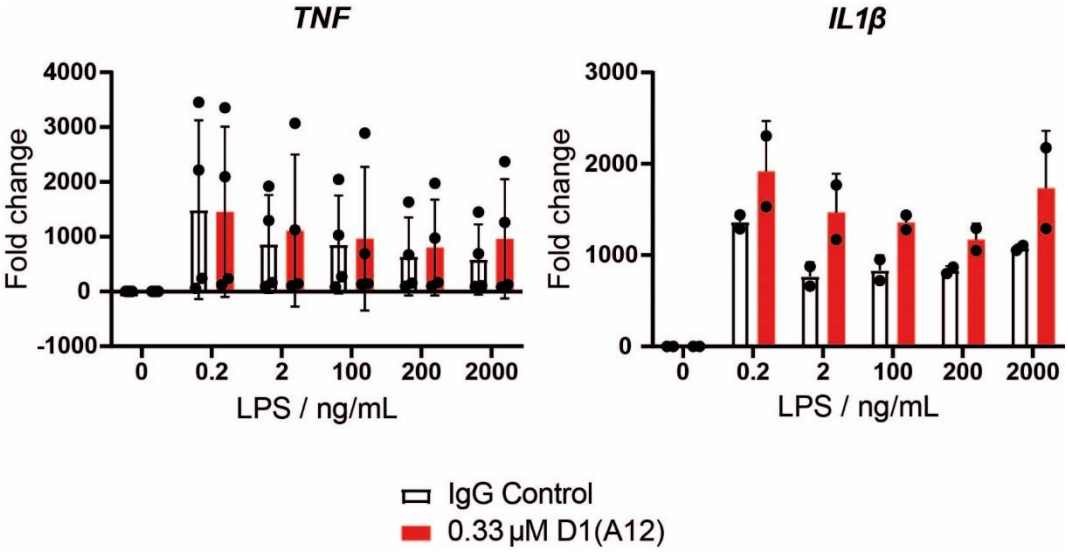

Figure 5.8 Inhibiting ADAM17 had no effect on LPS-stimulated changes in gene expression.

Primary human macrophages were pre-incubated for 1 h with D1(A12) blocking antibody (0.33 µM) or the same concentration of human IgG. Cells were stimulated with LPS at a range of concentrations (0.2 – 2000 ng/mL) for 6 h in combination with the blocking antibody. RNA was isolated and reverse transcribed to cDNA. Relative gene expression was calculated by qRT-PCR relative to the housekeeper *GAPDH* and fold change was compared relative to cells at 0 h time point. IgG control vs D1(A12) treated pairs were analysed using multiple Mann-Whitney U tests (mean ± SD, 4 independent donors for *TNF*, 2 independent donors for *IL1β*).

5.3.2 siRNA knockdown of *ADAM17*

Alongside the small molecule inhibitors and the blocking antibody, I also tried to use siRNA targeting of *ADAM17* to knockdown gene expression as an alternate method of inhibiting ADAM17 activity. I used electroporation to transfect the primary macrophages with the *ADAM17*-targeting siRNA (200 nM) and non-targeting siRNA (200 nM) as a control. I measured the expression of *ADAM17* 24 and 48 h after electroporation by qRT-PCR relative to a housekeeper gene, *GAPDH* (Figure 5.9), but found the *ADAM17*-targeting siRNA did not significantly reduce *ADAM17* expression relative to the non-targeting control at both time points. The reduction that was observed was maintained even when the cells were stimulated with LPS (100 ng/mL) for 6 h following 24 h of culture post-electroporation.

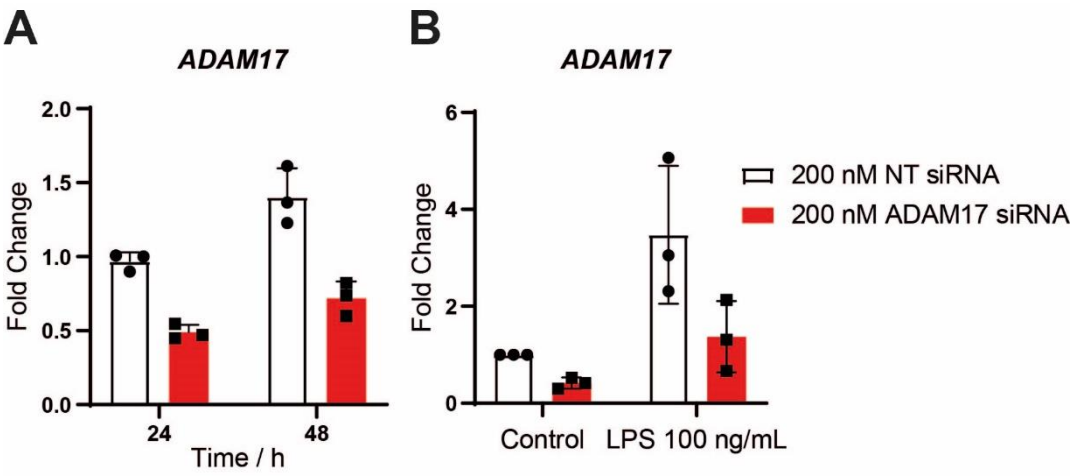

Figure 5.9 Expression of *ADAM17* was not significantly reduced by siRNA knockdown.

Primary human macrophages were electroporated either with siRNA targeting *ADAM17* (200 nM) or with non-targeting control siRNA. (A) Cells were cultured for 24 or 48 h before RNA was isolated and reverse transcribed to cDNA. Relative expression of *ADAM17* was calculated by qRT-PCR relative to the housekeeper *GAPDH*. Fold change was compared relative to non-targeting control cells after 24 h of culture. (B) 24 h post-transfection, cells were stimulated with LPS (100 ng/mL) for 6 h. RNA was isolated and reverse transcribed to cDNA before relative expression of *ADAM17* was calculated by qRT-PCR relative to the housekeeper *GAPDH*. Fold change was compared relative to the unstimulated non-targeting control. Data were analysed using multiple Mann-Whitney U tests (mean ± SD, 3 independent donors).

Due to time constraints, I was only able to investigate how knocking down *ADAM17* affected LRP1 shedding in macrophages from two donors. 24 h post-electroporation, I stimulated the macrophages with LPS (100 ng/mL) for 6 h and analysed the conditioned media by western blotting for LRP1 (Figure 5.10). In both donors, I did not observe LPS-stimulated shedding of LRP1 and the effect of *ADAM17* knockdown was inconsistent. In donor 1, the knockdown had no effect on the levels of LRP1 in the medium regardless of LPS stimulation, while in donor 2, *ADAM17* knockdown reduced LRP1 levels in the LPS-stimulated condition compared to the non-targeting control. I also found that the knockdown had no effect on LPS-stimulated release of TNF in either donor. Additionally, even in the non-targeting siRNA transfected controls, the amount of TNF released was very low compared to previous experiments (Figure 5.6) which used a similar cell number and time of LPS stimulation. In donor one, the amount of TNF released was so low that it was not accurately detectable by the ELISA, leading to high variation in technical replicates. This indicates that electroporation significantly impacted macrophage viability, so I did not pursue this approach further.

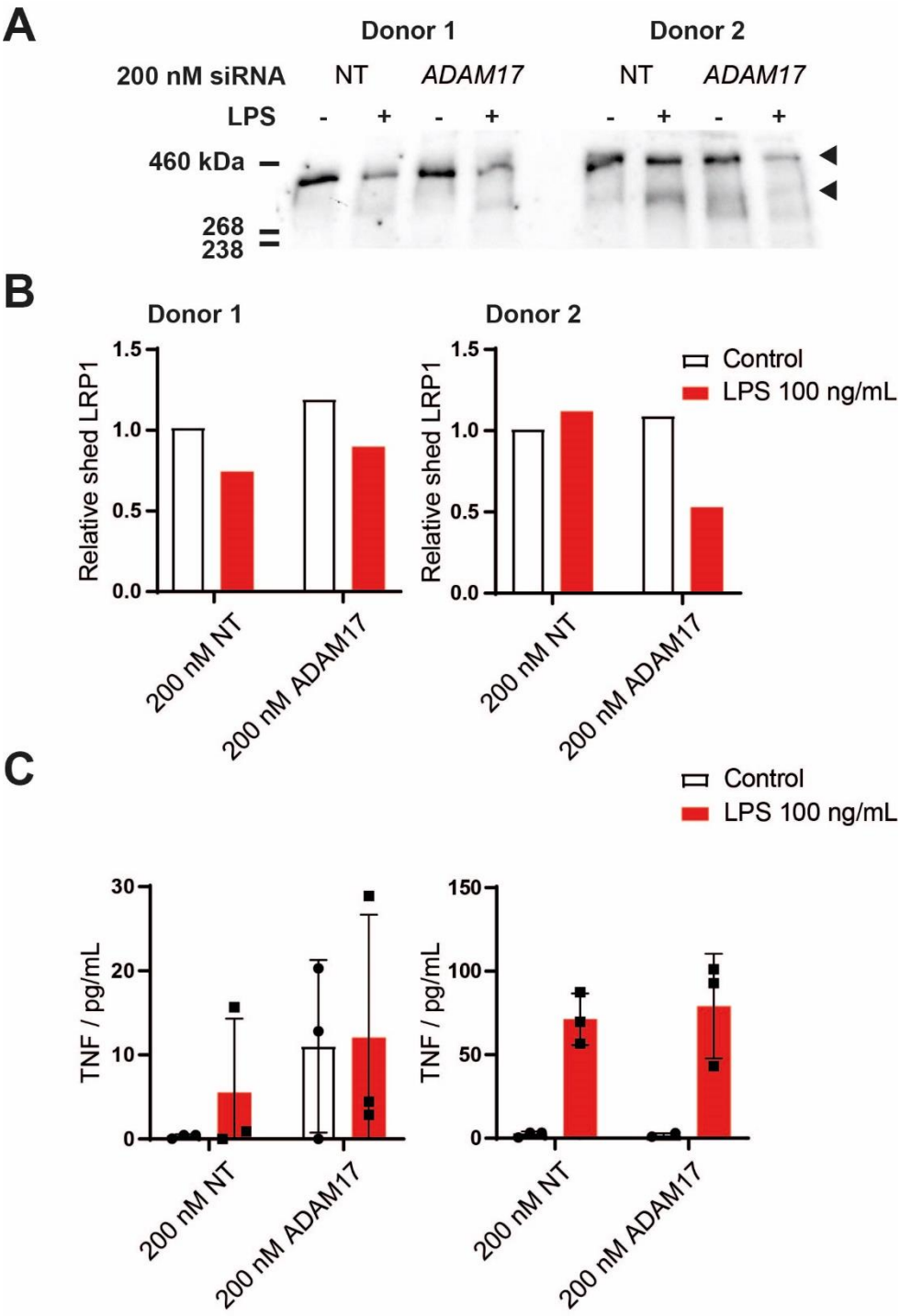

Figure 5.10 Knockdown of *ADAM17* by electroporation with siRNA impaired macrophage cellular responses.

Primary human macrophages were electroporated either with siRNA targeting *ADAM17* (200 nM) or with non-targeting control siRNA. 24 h post transfection, cells were stimulated with LPS (100 ng/mL) for 6 h. 2 independent donors are shown separately due to differences in effect. Conditioned media were collected and concentrated with TCA before analysis by SDS-PAGE and immunoblotting for LRP1 (A). Bands (◄) were quantified and normalised against a Revert Total Protein Stain and shed LRP1 levels calculated relative to the unstimulated non-targeting control (B). TNF concentration in conditioned media was

analysed by ELISA (C). Data were analysed using multiple Mann-Whitney U tests (mean ± SD, 3 technical replicates).

5.4 Selective inhibition of ADAM10 with the hydroxamate GI 254023X

Following on from experiments showing that ADAM17 is not involved in the shedding of LRP1 in primary human macrophages (Section 5.3.1), I decided to investigate ADAM10. I used the selective ADAM10 hydroxamate inhibitor GI 254023X to do this as it has a much higher potency for ADAM10 than it does ADAM17, with an IC50 of 5.3 nM for ADAM10 and 541.0 nM for ADAM17 (Hundhausen et al., 2003).

I first quantified the effect of 6 h of GI 254023X treatment on cell viability using an MTS assay (Figure 5.11A). Cell number was not significantly reduced by treatment with 1 μM GI 254023X, and there was not any significant decrease in cell number at 5 μM GI 254023X (by around 25%). I decided to use the higher concentration of GI 254023X going forward, as when it has been previously used, optimal inhibition of ADAM10 was only achieved at concentrations higher than 1 μM (Moss et al., 2007).

I also quantified TNF release by ELISA in the conditioned media of macrophages which were treated with GI 254023X (5 μM) and LPS-stimulated (Figure 5.11B). I found that ADAM10 inhibition had no effect on TNF release regardless of the concentration of LPS used, confirming that GI 254023X is selective for ADAM10 over ADAM17.

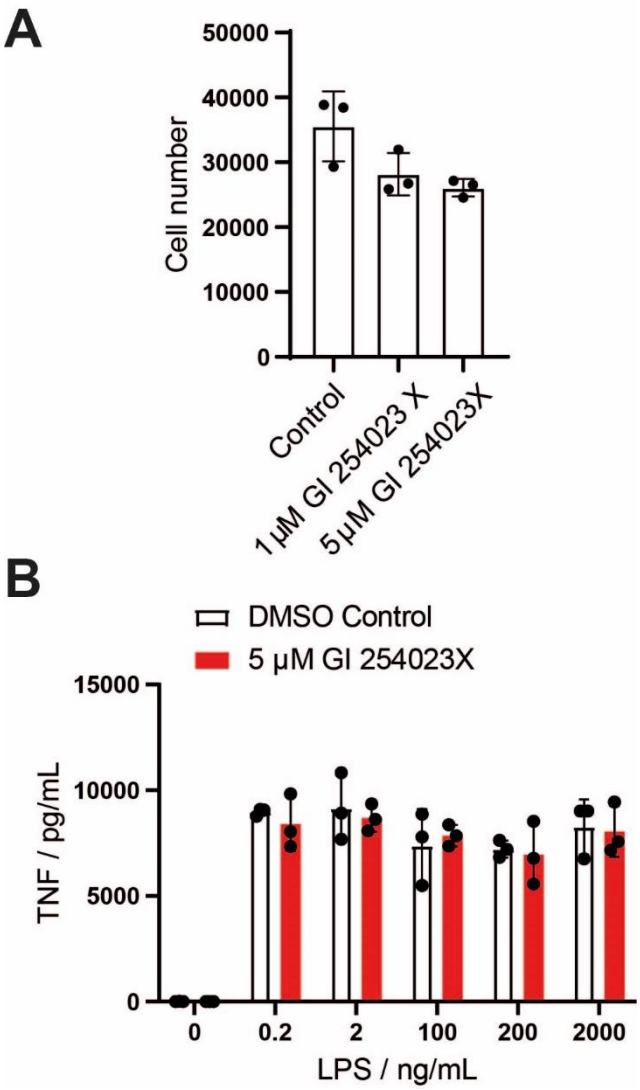

Figure 5.11 Inhibiting ADAM10 had no effect on LPS-stimulated TNF release and no significant effect on cell viability.

(A) Primary human macrophages were treated with GI 254023X (1 or 5 µM) for 6 h. Cell number was quantified using an MTS assay. Data were analysed using a Kruskal-Wallis and corrected for multiple comparisons with Dunn's test (mean ± SD, 3 technical replicates on 1 donor). (B) Primary human macrophages were pre-incubated for 1 h with GI 254023X (5 µM) or an equal volume of DMSO as a control. Cells were stimulated with LPS at a range of concentrations (0.2 – 2000 ng/mL) for 6 h in combination with the inhibitor. TNF concentration in conditioned media was analysed by ELISA. Data were analysed using multiple Mann-Whitney U tests (mean ± SD, 3 independent donors).

I found that pre-incubation with GI 254032X significantly reduced levels of LRP1 in the conditioned media of both unstimulated and LPS stimulated (0.2 ng/mL) macrophages (Figure 5.12). ADAM10 inhibition reduced constitutive shedding of LRP1 by 59% and LPS-stimulated shedding by 40%. At further doses of LPS (0.2 – 2000 ng/mL), where I only had three replicates, I was not able to detect any significant decrease in LRP1 shedding. From

this I concluded that ADAM10 played role in the shedding of LRP1 although it was unclear whether it was a purely constitutive role, or if it was also responsible for LPS-stimulated shedding of LRP1.

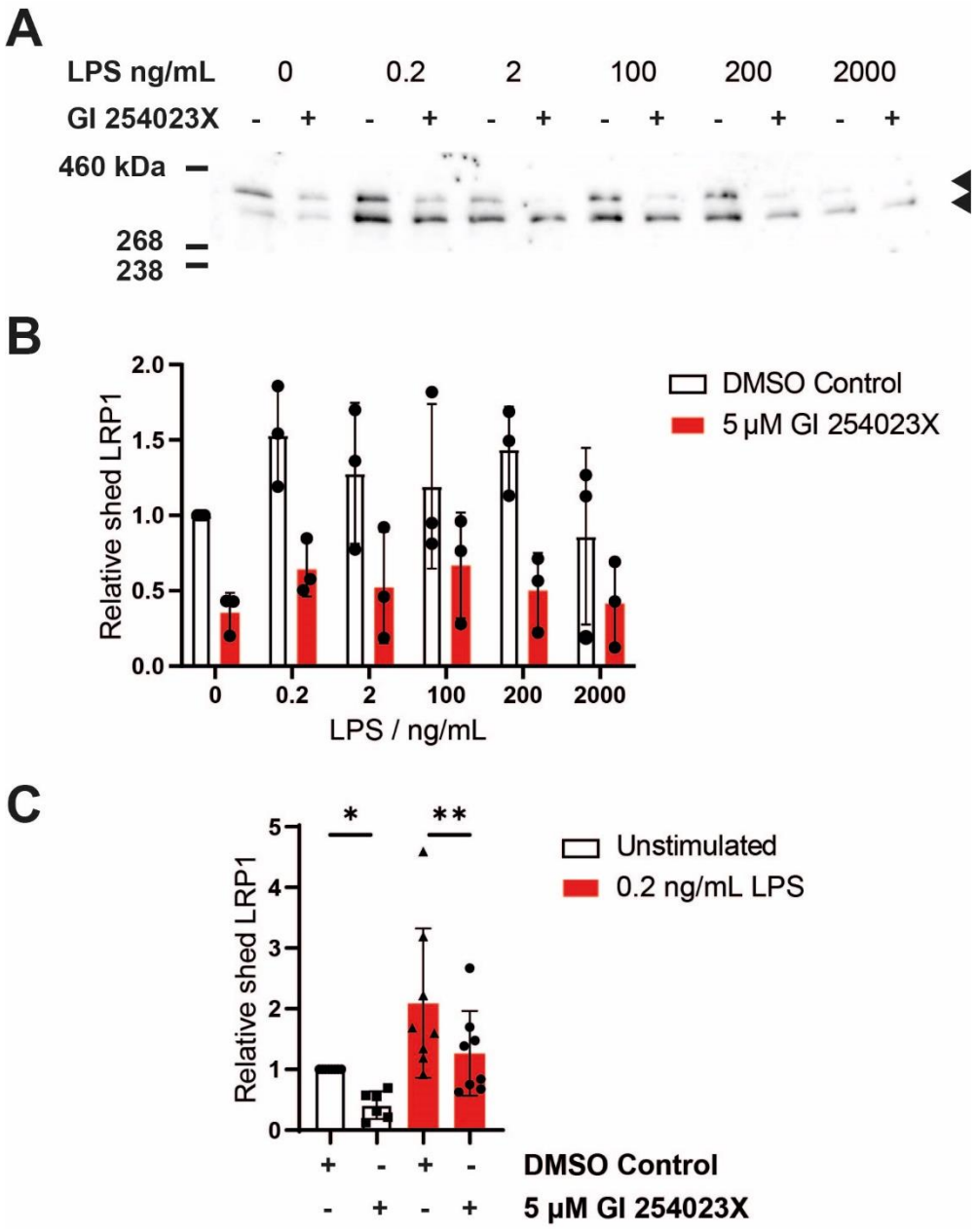

Figure 5.12 Inhibiting ADAM10 significantly reduced both constitutive and LPS stimulated LRP1 shedding.

Primary human macrophages were pre-incubated for 1 h with GI 254023X (5 µM) or an equal volume of DMSO as a control. Cells were stimulated with LPS at a range of concentrations (0.2 – 2000 ng/mL) for 6 h in combination with the inhibitor. Conditioned media were collected and concentrated with TCA before analysis by SDS-PAGE and immunoblotting for LRP1 (A). Bands (◄) were quantified and normalised against a Revert Total Protein Stain and shed LRP1 levels calculated relative to the unstimulated control (B and C). Data were analysed using multiple Mann-Whitney U tests (mean ± SD, 3

independent donors, * $p < 0.05$, ** $p < 0.01$). Further independent donors were used for LPS stimulation at 0.2 ng/mL (8 independent donors).

5.5 Stimulation of ADAM10 with ionomycin increased LRP1 shedding

After demonstrating that inhibition of ADAM10 inhibited constitutive and LPS-stimulated LRP1 shedding (loss of function), I wanted to investigate whether stimulating ADAM10 activity via an alternate mechanism could increase the shedding of LRP1 (gain of function). To do this I used the calcium ionophore ionomycin which activates ADAM10 through elevating cytosolic Ca^{2+} levels (Bleibaum et al., 2019; Horiuchi et al., 2007; Le Gall et al., 2009; Maretzky et al., 2015).

I used an MTS assay to quantify the effect of a range of concentrations of ionomycin on cell viability. The ionomycin was reconstituted in DMSO, so an equivalent volume of DMSO for each concentration of ionomycin was used a control. I only observed an effect on cell viability at high concentrations of both DMSO Ionomycin. To check for any stimulation of ADAM17, I measured TNF release into the conditioned media by ELISA (Figure 5.13). I found that ionomycin treatment did not stimulate TNF release and also did not alter LPS-stimulated TNF release which showed that using these conditions it did not stimulate ADAM17.

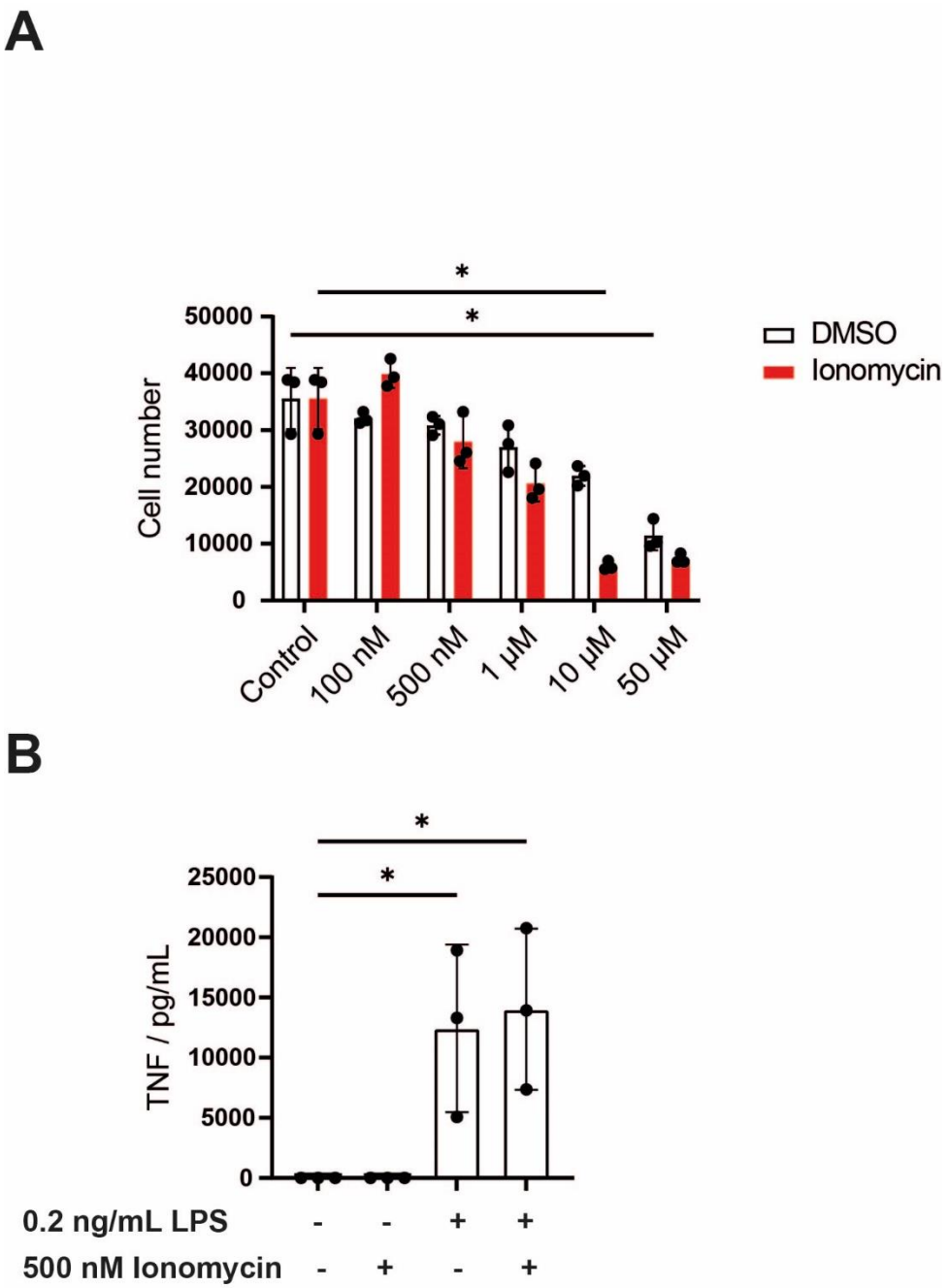

Figure 5.13 Ionomycin had no effect on TNF release only reduced cell viability at high concentrations.

(A) Primary human macrophages were treated with ionomycin at a range on concentrations (100 nM – 50 µM) or an equivalent volume of DMSO as a control for 6 h. Cell number was measured using an MTS assay. Data were analysed using multiple Mann-Whitney U tests (mean ± SD, 3 technical replicates, * $p < 0.05$). (B) Primary human macrophages were stimulated with either LPS (0.2 ng/mL), ionomycin (500 nM), or both for 6 h. TNF concentration in conditioned media was analysed by ELISA. Data were analysed using a Kruskal-Wallis corrected for multiple comparisons with Dunn's test (mean ± SD, 3 independent donors, * $p < 0.05$).

The concentration of ionomycin widely used in the literature is 2.5 μM (Maretzky et al., 2015), but I proceeded to test the effect of 500 nM ionomycin on LRP1 shedding as I did not want to greatly affect the cell viability.

Treatment with ionomycin caused a large increase in total protein release from the macrophages into the conditioned media as shown by the Revert Total Protein Stain (Figure 5.14). This was mirrored in the western blot for LRP1 by an increase in the levels of shed LRP1 after both 1 and 6 h of ionomycin treatment. After 1 h of ionomycin treatment, the increase of LRP1 shedding was not affected by ADAM10 inhibition. However, at 6 h the ionomycin-induced increase in LRP1 shedding was completely inhibited by pre-incubation of the cells with GI 254023X. These data further strengthened my conclusion that ADAM10 is the primary LRP1 sheddase in primary human macrophages.

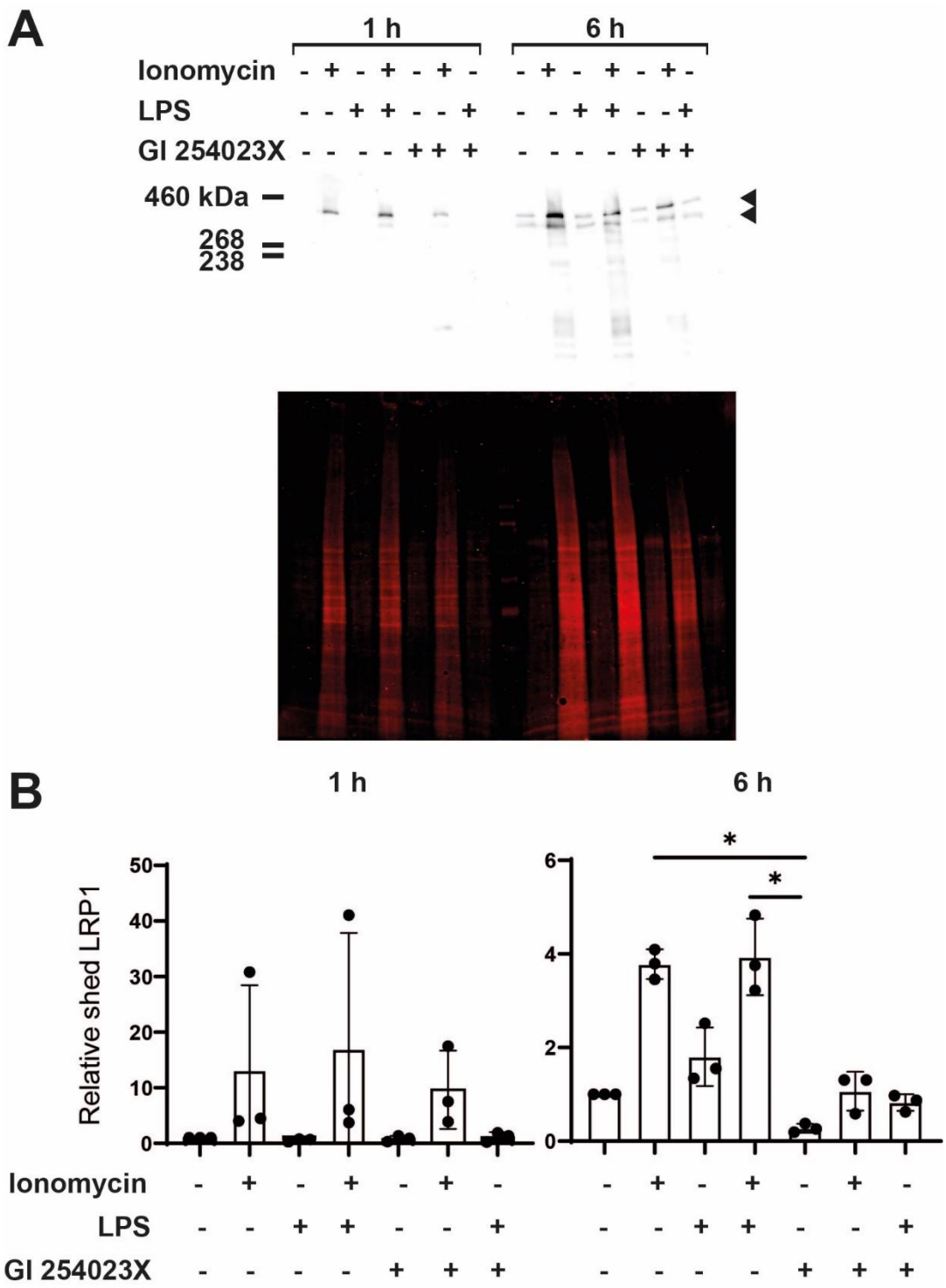

Figure 5.14 Treatment with Ionomycin increased shedding of LRP1 which was inhibited by pre-incubation with GI 254023X.

Primary human macrophages were pre-incubated for 1 h with GI 254023X (5 µM) or DMSO as a control. Cells were stimulated with either LPS (0.2 ng/mL), ionomycin (500 nM), or both, for 1 or 6 h in combination with the inhibitor. Conditioned media were collected and concentrated with TCA before analysis by SDS-PAGE and immunoblotting for LRP1 (A). Revert Total Protein Stain of the membrane is also shown below the western blot. Bands (◄) were quantified but not normalised against the Revert Total Protein Stain and shed LRP1 levels calculated relative to the unstimulated control (B). Data were analysed using a

Kruskal-Wallis and corrected for multiple comparisons with Dunn's test (mean ± SD, 3 independent donors, * $p < 0.05$).

5.6 Profiling expression of the C8 tetraspanins in primary human macrophages

Following my conclusion that ADAM10 plays a key role on the shedding of LRP1 from primary human macrophages (Sections 5.4 and 5.5), I wanted to investigate how ADAM10 may be regulated in human macrophages.

The C8 family of tetraspanins are proteins that interact with ADAM10 and regulate its trafficking, localisation, and substrate specificity (Harrison et al., 2021). I measured the expression of the TspanC8s in primary human macrophages after M-CSF treatment but prior to any further stimulation (0 h time point) and found that while *TSPAN10* was not detectable and *TSPAN5* was very weakly expressed, the other four TspanC8s were expressed reasonably highly (Figure 5.15). I therefore looked at how the expression of those four tetraspanins changed during 24 h of LPS stimulation. As I only had two independent replicates of this, I was unable to do any statistical tests of significance on the data, however, these preliminary data do show some clear trends. *TSPAN14* expression was upregulated in response to LPS at 4 and 6 h but returned to basal expression levels by 24 h. *TSPAN15* expression was progressively down regulated over 24 h in both the control and LPS-stimulated conditions, but the downregulation was more marked in the presence of LPS. *TSPAN17* expression was steady over 24 h, with slight downregulation in response to LPS. *TSPAN33* expression was strongly upregulated by LPS stimulation, reaching a peak at 12 h.

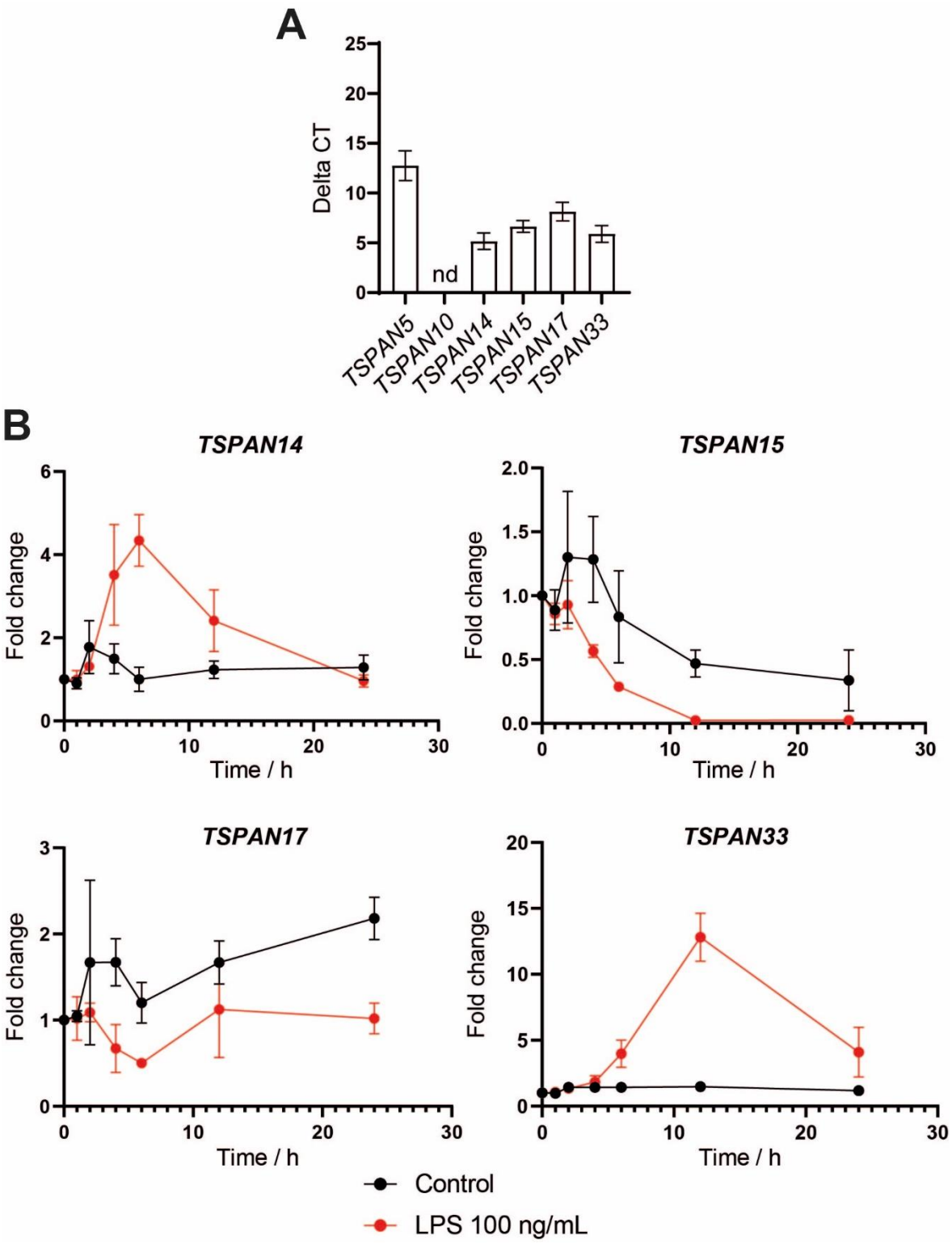

Figure 5.15 LPS stimulation changed the expression of the C8 tetraspanins over time. Primary human macrophages were stimulated with LPS (100 ng/mL) or control media for 24 h. RNA was isolated at time points (1, 2, 4, 6, 12, and 24 h) and reverse transcribed to cDNA. (A) Delta CT values of the C8 tetraspanin genes from cells at 0 h (mean ± SD, 5 independent donors). (B) Relative gene expression was calculated by qRT-PCR relative to the housekeeper *GAPDH* and fold change was compared relative to cells at 0 h time point (mean ± SD, 2 independent donors).

5.7 Discussion

In this chapter I aimed to investigate which protease or proteases are responsible for the constitutive and LPS-stimulated shedding of LRP1 from primary human macrophages. Initially I tested how LPS-stimulated shedding of LRP1 was affected by a range of broad-spectrum protease inhibitors targeting the major classes of protease. As ADAM17 was my primary candidate I included several metalloprotease inhibitors along with inhibitors of serine, cysteine, and aspartyl proteases. I found that none of the inhibitors tested significantly decreased either the constitutive or the stimulated shedding of LRP1. However, the lack of statistical significance was impacted by the variation in effect size of LPS stimulation on macrophages from different donors, so I also considered whether non-significant trends were evident. Treatment with two of the metalloprotease inhibitors tested, TAPI-2 and GM6001, along with the cysteine protease inhibitor E-64, resulted in a trend towards reduced LRP1 shedding. With further replicates or optimisation of the treatment conditions I would potentially have been able to observe significant changes. But even without significance, the results indicated possible involvement of one or more metalloproteases and cysteine proteases. All the protease inhibitors tested did have an effect on cell viability however they all had very similar effects, and none of them reduced viability by more than 37% so I decided that the treatment conditions could still be compared to each other.

It is unclear why treatment with AEBSF caused the large increase in LPS stimulated total protein release and LRP1 release. Due to its effects on total protein release, it is unclear whether AEBSF directly increased LRP1 shedding or whether the observed increase in LRP1 is a consequence of a more generalised effect on protein release. While AEBSF did have an effect on cell viability, this was comparable with the other inhibitors evaluated so it is unlikely that the substantial protein release was caused by release of cellular contents after cell death. I also centrifuged conditioned media from AEBSF-treated cells prior to TCA concentration to try and remove any cell membrane debris, but this had no effect on the resultant Revert Total Protein Stain and western blot. The lack of effect on non-LPS-stimulated cells also suggests that this was a response caused by the combination of AEBSF and LPS rather than any toxicity from the AEBSF. This effect was not replicated when PMSF, another serine protease inhibitor, was tested, suggesting that the increase in protein release was caused by an effect of AEBSF other than its serine protease inhibitory effects. Other mechanisms of action have been reported for AEBSF such as inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation (Diatchuk et al., 1997) which are not shared by other serine protease inhibitors. However, the data indicate that serine proteases are unlikely to mediate LRP1 shedding, and investigation of the effects of AEBSF on protein release were beyond the scope of my project.

I measured LPS-stimulated TNF release in cells treated with the full range of protease inhibitors to both confirm selectivity of the inhibitors towards ADAM17 and as an indirect measure of cell viability. Due to variation in the amount of TNF released by individual donors, I calculated the relative TNF release compared to the LPS-stimulated control for each donor. This allowed for better comparison, because while the actual concentrations of TNF released varied widely, the trends between the groups were consistent. The metalloprotease inhibitors GM6001 and CT1746 significantly decreased TNF release as expected given their ability to inhibit ADAM17 (English et al., 2000; Moss & Rasmussen, 2007). The other metalloprotease inhibitor TAPI-2 did reduce TNF release but not significantly suggesting that it was not as effective at targeting ADAM17 as GM6001 and CT1746 under these conditions. The fact that CT1746 was the most potent inhibitor of TNF release suggests that it was a better inhibitor of ADAM17 in this cell type than GM6001 and TAPI-2. This does not fit with their K_i values as GM6001 has the highest in vitro potency (Moss & Rasmussen, 2007). There are a number of possible reasons for this. The inhibitors are all low molecular weight hydroxamate peptides and so could be endocytosed by the cells and broken down, but at different rates. If an inhibitor also has other targets which are highly expressed in this cell type, then that could reduce its inhibition of ADAM17. It is also possible that the concentrations used for each inhibitor were not optimal for this cell type.

However, CT1746 had no effect on LPS-stimulated LRP1 shedding while the other two metalloprotease inhibitors did. This was the first indication that a metalloprotease other than ADAM17 is responsible for LRP1 shedding. However, metalloproteases have very similar catalytic domains which means that none of these hydroxamate inhibitors are specific for a particular metalloprotease. This makes it difficult to unequivocally identify a role for any specific metalloprotease using hydroxamate inhibitors.

I therefore also used more specific inhibitors of ADAM17 that have reduced off-target inhibition of other metalloproteases. The first method I used was the cross-domain human ADAM17 blocking antibody D1(A12) IgG1 (Tape et al., 2011). The antibody achieves high specificity for ADAM17 by binding to the enzyme in two places, namely the ADAM17-specific noncatalytic Disintegrin and Cysteine-rich domains and the catalytic domain, which enables the antibody to inhibit ADAM17 in a highly selective manner.

I found that treatment with D1(A12) had no effect on constitutive or LPS-stimulated shedding of LRP1, irrespective of LPS concentration used. This provided strong evidence that ADAM17 is not involved in the shedding of LRP1 from human macrophages. D1(A12) was able to partially block TNF release but this was only significant at low concentrations of LPS. TNF release was still reduced by D1(A12) at higher LPS concentrations, with the lack of significance possibly be due to variation between the donors rather than a lack of efficacy.

In order to further rule out ADAM17 as being involved in LRP1 shedding in human macrophages, I wanted to use siRNA knockdown of *ADAM17*. Unfortunately, the optimisation of this in primary macrophages proved challenging. Using electroporation to transfect the siRNA into the macrophages, I was able to knockdown *ADAM17* by roughly 50% and maintain this for 48 h in culture. However, this process often resulted in cell numbers too low to detect LRP1 by western blotting at the time point I was interested in. Consequently, I was only able to measure the effect of *ADAM17* knockdown on LRP1 shedding in cells from two donors, both of which responded poorly to LPS and released low levels of TNF, indicating electroporation had a marked effect on cellular responses and viability. With more time I would have liked to further optimise the knockdown of *ADAM17* and then *ADAM10* as it is the best way to ensure specificity of inhibition.

An alternative method for investigating this question would have been to use clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 knockout of *ADAM17* and *ADAM10* (separately and in combination) in induced pluripotent stem cells (iPSCs) with subsequent differentiation to macrophages. However, this was not possible due to time constraints. Despite these limitations, I was confident that experiments with the D1(A12) ADAM17 blocking antibody showed that a metalloprotease other than ADAM17 was primarily responsible for LRP1 shedding in human macrophages. Therefore, I next investigated the effect of inhibiting ADAM10 on LRP1 shedding.

ADAM10 and 17 are very closely related to each other (Edwards et al., 2009) and so ADAM10 was the logical next candidate to investigate. I inhibited ADAM10 with GI 254023X, a hydroxamate inhibitor shown to be 100-fold more effective at inhibiting ADAM10 than ADAM17 (Hundhausen et al., 2003). When I inhibited ADAM10 with GI 254023X, both the constitutive and stimulated shedding of LRP1 was significantly reduced. I observed this reduction at all concentrations of LPS investigated, although the effect was not significant at 100 and 2000 ng/mL LPS. Despite the small but significant effect on cell viability caused by the concentration of GI 254023X I tested, there was no effect on TNF release. This suggests that any reduction in cell number was not large enough to result in a detectable difference in how the cells responded to LPS stimulation. This provides evidence to show that the reduction in shed LRP1 levels was not due to a reduced number of cells shedding LRP1, and instead due to inhibited shedding.

GI 254023X inhibited LRP1 shedding under basal as well as LPS-stimulated conditions, so it is unclear whether ADAM10 is involved in purely constitutive shedding, or both constitutive and LPS-stimulated shedding. Additionally, ADAM10 inhibition with GI 254023X did not completely abolish basal or LPS-stimulated shedding of LRP1, so other protease(s) could be involved in the shedding process. For example, E-64 showed some inhibition of LRP1 shedding, indicating a cysteine protease may contribute to overall levels of LRP1 cleavage.

Alternatively, the incomplete inhibition observed may reflect some remaining ADAM10 activity at the concentration of GI 254023X used.

GI 254023X treatment had no effect on TNF release regardless of LPS concentration used demonstrating that it is specific for ADAM10 and has no off-target inhibition of ADAM17. This supports previous characterisation of this inhibitor by Hundhausen *et al.* (2003). This role of ADAM10 is further supported by the results of ionomycin treatment. Ionomycin either on its own or in combination with LPS did not affect TNF release showing that it specifically stimulates ADAM10 without any targeting of ADAM17.

Ionomycin caused a large increase in LRP1 levels in the conditioned media along with an increase in the total protein released from the cells. At the 6 h time point the increase in LRP1 was inhibited by co-treatment with GI 254023X while maintaining the large total protein release. This demonstrated that the increased levels of LRP1 in the media were because of ADAM10 stimulation rather than a consequence of the general protein release. However, this was not the case at the 1 h time point. One reason for this could be that the ADAM10 inhibitor needs longer to have an effect and maybe 1 h of pre-incubation is not long enough to fully inhibit ADAM10. Another possibility could be that at 1 h the increase in LRP1 is due to the increased total protein release and that when stimulated with ionomycin, the ADAM10 mediated shedding of LRP1 is a slower process. The slight reduction in cell viability I observed at the concentration of ionomycin used (500 nM) was not responsible for the increased total protein release as even at lower concentrations, where there was no effect on cell viability, total protein release was still increased. Together with the inhibition of ADAM10 with GI 254023X, this demonstrates both loss and gain of function of ADAM10 modulates LRP1 shedding, providing strong evidence for the role of ADAM10 in the shedding of LRP1 from human macrophages.

Following on from this, I wanted to investigate how ADAM10-mediated shedding of LRP1 could be regulated. ADAM10 associates with members of the TspanC8 family of tetraspanins which regulate ADAM10 trafficking from the ER to the cell membrane (Dornier et al., 2012), as well as the substrate selectivity of the protease at the cell membrane (Jouannet et al., 2016), and then its endocytosis (Eschenbrenner et al., 2020). Matthews *et al.* (2018) consider that there are six distinct forms of ADAM10 each with different substrate specificities, depending on which of the TspanC8s ADAM10 associates with. A recently hypothesised mechanism for this is that the TspanC8s regulate the distance above the membrane at which the ADAM10 active site cleaves, hence controlling which substrate cleavage sites it can target (Koo et al., 2022).

Of the six TspanC8s, *TSPAN*5 and *10* were not expressed in unstimulated primary human macrophages but the other four were expressed at roughly similar levels. *TSPAN14* and

TSPAN33 were expressed marginally more than *TSPAN15* and *TSPAN17*. This is consistent with previously reported data on the expression profile of the TspanC8s in macrophages (Harrison et al., 2021). The regulation of ADAM10 substrate specificity by the TspanC8s could explain the differences in how LRP1 is shed in different cell types, since different cell types have different profiles of tetraspanin expression, and so give rise to varying ADAM10 substrate repertoires dependent on TspanC8 expression in the different cell types. There are also differences in the expression of the TspanC8s between human and murine leukocytes (Matthews et al., 2018), which could be the reason that ADAM10 sheds LRP1 in human macrophages while ADAM17 is the primary LRP1 sheddase in murine macrophages (Gorovoy et al., 2010).

My preliminary data showed that the expression of the tetraspanins changed in response to LPS stimulation and that each of the four expressed tetraspanins had different responses. *TSPAN33* was highly upregulated in response to the LPS stimulation over the time course investigated, while the expression of *TSPAN15* was strongly downregulated. This could suggest that the tetraspanin which is associated with ADAM10 changes in response to LPS stimulation. If this was the case, it would allow for the precise regulation of how ADAM10 targets LRP1 for shedding. When associated with Tspan33 for example, ADAM10 might be able to target LRP1 better than if associated with Tspan15. The upregulation of *TSPAN33* was first detectable from 6 h, the time point at which I could reliably detect LPS-stimulated LRP1 shedding. This correlation could suggest that further investigation is warranted to determine whether Tspan33 may support the ADAM10-mediated shedding of LRP1, and whether upregulation of *TSPAN33* gene expression serves as a feed-forward mechanism to increase LRP1 shedding in response to LPS stimulation.

It was unclear from my results whether ADAM10 shedding of LRP1 is purely constitutive or is also able to be stimulated by LPS. Potentially, investigating how ADAM10 associates with the TspanC8s in this model could help to elucidate this.

Chapter 6: Final Discussion

6.1 Regulation of LRP1 shedding in macrophages is important for regulation of the inflammatory response

The release of TNF from macrophages is an important step in the mechanism of defence against infection and tissue damage, as TNF induces expression and release of other pro-inflammatory cytokines, and so initiates the pro-inflammatory cascade characteristic of the acute inflammatory response (Feldmann, 2002; Murphy & Weaver, 2016). Elevated and prolonged TNF release is associated with several diseases such as rheumatoid arthritis (Feldmann et al., 1995) and Crohn's disease (van Dullemen et al., 1995). This indicates tight regulation of TNF release from macrophages is of great importance for a healthy inflammatory response.

The precursor form of TNF is a transmembrane trimeric protein that is cleaved by the metalloprotease ADAM17 to release active TNF from the cell in response to inflammatory stimuli (Black et al., 1997). The activity of ADAM17 is primarily regulated rapidly and reversibly at the cell surface upon stimulation (le Gall et al., 2010), and a number of mechanisms have been proposed to explain this, as detailed previously (Section 1.2.2). There has been a lot of research into how ADAM17 can be activated and how that activation is controlled, but there is much less research concerning how ADAM17 activity is terminated and homeostasis restored. One mechanism known to reduce ADAM17 activity is its inhibition by TIMP-3 at the cell surface (Amour et al., 1998; Black, 2004). This acts as a crucial method of terminating TNF release following the initial inflammatory response. Levels of TIMP-3 on the cell surface are regulated by endocytosis and lysosomal breakdown by LRP1 in both chondrocytes (Doherty et al., 2016; Scilabra et al., 2013) and macrophages (Schubert et al., 2019) demonstrating a further level of regulating TNF release.

LRP1 can be proteolytically cleaved in the juxta-membrane region of its β-chain, releasing a soluble form of the protein consisting of part of the β-chain non-covalently coupled to the α-chain (Grimsley et al., 1998; Quinn et al., 1999). This shedding reduces the cell surface levels of LRP1 and so reduces cellular capacity to endocytose LRP1 ligands, and also produces a soluble form of LRP1 that acts as a decoy receptor by competing with cell surface LRP1 for ligand binding (Gorovoy et al., 2010; Scilabra et al., 2013). Xu *et al.* (2014) proposed that in resting cells, ADAM17 and TIMP-3 form an inactive complex on the cell surface, and that this complex dissociates upon LPS stimulation, releasing ADAM17 to cleave pro-TNF. Schubert *et al.* (2019) built on this model, proposing that the dissociated TIMP-3 is endocytosed by LRP1 in the early stages after LPS stimulation, but that LPS also stimulates shedding of LRP1, causing TIMP-3 to progressively accumulate on the cell

surface, thereby inhibiting ADAM17 activity and TNF release, and so resolving the inflammatory response.

However, the mechanism by which LRP1 is shed is poorly understood, especially in human macrophages. Various LRP1 ‘sheddases’ have been proposed, often depending on the cell type in which the investigations were done. Metalloproteases are the most common class of protease suggested as LRP1 sheddases, and in the murine macrophage cell line RAW 264.7, ADAM17 was identified as the key LRP1 sheddase (Gorovoy et al., 2010). I thus hypothesised that this was the same in human macrophages, and that ADAM17 was involved in its own regulation, with ADAM17 activity initiating a negative feedback loop by shedding LRP1 in response to LPS-stimulation at a slower rate than its LPS-stimulated shedding of TNF. Therefore, I aimed to investigate which protease(s) are responsible for LRP1 shedding from human macrophages, and how this process may be controlled to regulate termination of TNF release and so resolution of the inflammatory response.

6.2 Monocyte-derived macrophages were a better model system than monocytic cell lines for investigating LRP1 shedding in human macrophages in vitro

Ideally, I would have liked to be able to first investigate LRP1 shedding in cell lines before moving on to analysis in primary cells. Cell lines have near unlimited availability, are easy to culture, and are inexpensive when compared with primary cells isolated from peripheral blood. Primary cells are also much more variable in their responses to biological stimuli due to each donor having a distinct genetic background and inflammatory state at the time of donation. As cell lines are highly consistent in their biological responses, it makes them a useful tool to investigate biological mechanisms (Tedesco et al., 2018).

The THP-1 and U937 monocytic cell lines are frequently used to investigate macrophage function in vitro (Nascimento et al., 2022). Both cell lines are leukemic, which gives them many useful properties such as unlimited proliferation, but does mean that they respond differently to many biological stimuli compared with primary cells (Daigneault et al., 2010; Tedesco et al., 2018). The main differences between the two cells lines are their origin and how they were immortalised, but both can be differentiated into macrophage-like cells using PMA. There are differences between the resultant differentiated cells, but in the literature these cell lines are often used interchangeably (Nascimento et al., 2022). I decided to test both cell lines to see which might make a suitable model for investigating LRP1 shedding in macrophages.

Unfortunately, I found that neither cell line was appropriate for my investigations. I was unable to detect shed LRP1 in the conditioned media from PMA-differentiated U937 cells stimulated with LPS. This made any further investigation of LRP1 shedding in this cell type impractical.

THP-1 cells were an improvement in this regard, as I was able to detect shed LRP1 in the conditioned media of PMA-differentiated cells as early as 2 h after changing to serum-free media, but I was unable to induce stimulated shedding of LRP1 with a range of pro-inflammatory stimuli. LPS-stimulated shedding of LRP1 was an important requirement for any model system, since this is a well-described feature of LRP1 shedding in both murine and human primary macrophages (Gorovoy et al., 2010; Schubert et al., 2019). The lack of THP-1 response to LPS is most likely due to their low level of expression of *CD14,* a component of the LPS signalling complex (Bosshart & Heinzelmann, 2004). A possible way to overcome this problem might have been to stably transfect the THP-1 cells with human CD14 as previously described (Lee et al., 1992), but even with this approach, the response of the cells to LPS is not comparable to that of primary macrophages (Bosshart & Heinzelmann, 2016).

It is less clear why IFN-γ did not stimulate LRP1 shedding. Gorovoy *et al.* (2010) observed robust LRP1 shedding in response to IFN-γ in a murine cell line, so differences in cell type may underline this difference. I could have spent more time attempting to optimise this by testing a range on concentrations of IFN-γ, but the concentration I used (50 ng/mL) did stimulate TNF release and was higher than previously used by Gorovoy *et al.* (2010) (25 ng/mL), so I decided to pursue other avenues.

Another variable with use of the cell lines is the PMA differentiation step. There is no consistent approach for differentiating the cell lines with PMA in the literature, which makes comparison between studies difficult. Different protocols produce cells with different morphology and different responses to stimuli (Aldo et al., 2013; Baxter et al., 2020). I chose a protocol which had been frequently used previously, but did not extensively profile the differentiated cells after PMA treatment to confirm a macrophage-like phenotype. I confirmed a macrophage-like morphology and adherence to the cell culture dish visually. Consequently, I do not know how similar the PMA-differentiated cells I obtained were to primary cells. Potentially, if I had further optimised the PMA-differentiation of the cell lines, I may have generated cells that responded better to pro-inflammatory stimuli in terms of their LRP1 shedding. However, as there are a vast number of different protocols I could have tested, and even the best would only result in a simplified model of a primary macrophage (Tedesco et al., 2018), I decided that it would be better to focus on using primary human cells. I followed the approach described by Schubert *at al.* (2019) of isolating monocytes from human peripheral blood, and differentiating them in vitro to macrophages.

There is considerable discussion in the literature on the differences between macrophages generated from monocytes using either M-CSF or GM-CSF and how each of them then polarise into a pro- or anti-inflammatory phenotype (de Brito Monteiro et al., 2020; Jaguin et al., 2013; Lacey et al., 2012; Murray et al., 2014). GM-CSF is generally considered to produce a mixed population of dendritic cells and macrophages which are somewhat activated and differentiated (Schmid et al., 2010; Ushach & Zlotnik, 2016). Conversely, M-CSF is broadly considered to produce a purer population of macrophages that are not activated (Schmid et al., 2010). This has resulted in GM-CSF-differentiated macrophages often being termed “M1”-like and M-CSF-differentiated macrophages labelled as “M2”-like. However, this is a simplistic model which does not consider the broad range of phenotypes observed depending on how the macrophages are stimulated (Martinez & Gordon, 2014). This made M-CSF the appropriate choice for my investigations as I wanted to be able to investigate LRP1 shedding from macrophages at the basal level, and then to look at how this changed with stimulation. I validated that the monocyte-derived macrophages expressed expected macrophage markers and that they also polarised as expected to pro- and anti-inflammatory stimuli (Section 4.2.1).

M-CSF-differentiated primary macrophages had a much higher basal level of LRP1 shedding than the cell lines. This made them a much better model for investigating the molecular mechanism of LRP1 shedding, as I was able to detect shed LRP1 in the conditioned media at earlier time points, and with a stronger band intensity. This meant changes in LRP1 shedding were easier to detect. Polarisation of the macrophages into either phenotype (with LPS and IFN-γ or IL4 and IL-13) did not affect the shedding of LRP1 (Section 4.2.3). The effect of these combinations of stimuli on LRP1 shedding has not previously been investigated, although increased levels of LRP1 shedding have been reported in various chronic inflammatory states, including rheumatoid arthritis (Gorovoy et al., 2010) and atherosclerosis (de Gonzalo-Calvo et al., 2015) and I did observe increased LRP1 shedding in response to LPS alone. This may indicate that my in vitro model didn’t fully recapitulate in vivo macrophages or did not reflect a chronic inflammatory state. Alternatively, this may indicate that IFN-γ suppressed the LPS-induced LRP1 shedding, but further work would be needed to confirm this especially given the variability between donors in the magnitude of LPS-stimulated LRP1 shedding.

6.3 Identification of ADAM10 as a major LRP1 sheddase in human macrophages

6.3.1 Use of highly selective inhibitors indicated ADAM10, but not ADAM17, mediates shedding of LRP1 from human macrophages

My primary hypothesis was that ADAM17 was the protease responsible for stimulated shedding of LRP1 as observed in MEF and RAW 264.7 cells (Gorovoy et al., 2010; Liu et al., 2009). I used several approaches to inhibit ADAM17 activity, and demonstrated their efficacy against ADAM17 by their ability to reduce LPS-stimulated TNF release, but none of these approaches reduced the shedding of LRP1 at the time points I tested. From this I concluded that ADAM17 most likely does not have a role in the shedding of LRP1 from human macrophages. I cannot completely rule out involvement of ADAM17 as I was not able to fully optimise siRNA knockdown of *ADAM17* and *ADAM10* (separately and in combination) in the macrophages which would have provided more evidence to support this conclusion. Another option to further validate ADAM17 having no role would have been to use CRISPR/Cas9 to knockout *ADAM17* and *ADAM10* in iPSCs before differentiating them to macrophages (Yanagimachi et al., 2013). This approach also has limitations, in that iPSCs may not fully recapitulate primary macrophages. I was confident that I had enough evidence to discount ADAM17 as the sheddase and switched focus onto ADAM10 as an alternative target.

When I inhibited ADAM10, both constitutive and LPS-stimulated LRP1 shedding were decreased (Figure 5.12) and conversely when I stimulated ADAM10 with calcium influx, both constitutive and LPS-stimulated shedding LRP1 shedding were increased (Figure 5.14). I thus demonstrated that both loss- and gain-of-function of ADAM10 altered LRP1 shedding. I am not able to conclude from the data whether ADAM10 is purely a constitutive sheddase of LRP1, or whether it is also involved in the LPS-stimulated shedding. This is primarily because LPS stimulation increased LRP1 shedding by a modest amount (on average 2-fold, Figure 4.10), and additionally because ADAM10 inhibition/activation altered the basal and LPS-stimulated shedding of LRP1 by similar percentages.

ADAM10 is generally considered to be a constitutively active metalloprotease as opposed to ADAM17 which is more responsive to activating stimuli (Ludwig et al., 2005). Previous studies have shown that ADAM10 can be stimulated by either calcium influx or activation of the P2X7 receptor (Le Gall et al., 2009) but the exact mechanisms of activation are still unclear. ADAM10 is not generally thought to be directly stimulated by LPS like ADAM17 is, but the P2X7 receptor, which is an ATP-gated ion channel, is involved in inflammation (Adinolfi et al., 2018) and its activity is stimulated by LPS signalling (Yang et al., 2015). This

raises the possibility that ADAM10 may be activated downstream from LPS-stimulation rather than directly, which may explain why the ADAM10-mediated LPS-stimulated shedding of LRP1 that I observed here is slower than the ADAM17-mediated LPS-stimulated TNF release. However, the interplay between these signalling pathways is poorly understood so further investigation would be required to support this hypothesis.

My observation of ADAM10-dependent/ADAM17-independent LRP1 shedding in macrophages contrasts with previously reported results. ADAM10 has been identified (along with ADAM17) as an LRP1 sheddase in MEF cells (Liu et al., 2009), and ADAM17 alone was found to be responsible for LPS-stimulated shedding of LRP1 in a murine macrophage cell line (RAW 264.7). This difference is perhaps not surprising given the differences between mouse and human immunology (Mestas & Hughes, 2004). There are many similarities between the two, which is one of the reasons why using mice is such an important tool in biological research, but there are some important differences which need to be considered when comparisons are made. Importantly in this regard, human and murine macrophages polarise differently when treated with the same stimuli (Spiller et al., 2016) and have different metabolic responses to LPS (Vijayan et al., 2019).

The difference between ADAM10 and ADAM17 activity in human macrophages is interesting as the two metalloproteases are very closely related enzymes and share some of the same substrates (Edwards et al., 2009; Scharfenberg et al., 2020; Werny et al., 2022). They often also provide redundancy for each other, with ADAM10 being able to shed ADAM17 substrates when ADAM17 is absent (Le Gall et al., 2009). However, I observed that ADAM10 had a role in shedding LRP1 in human macrophages while ADAM17 did not. This increased level of complexity in LRP1 shedding in human macrophages (non-redundant ADAM10 and ADAM17 activity) compared to MEF cells (redundant ADAM10 and ADAM17 activity) could be because TNF release needs to be more tightly controlled in macrophages than in MEFs, as macrophages play a major role in regulation of inflammation.

I observed that inhibition of ADAM10 never completely blocked shedding of LRP1, suggesting either that ADAM10 is not the only protease responsible, or that I was unable to fully inhibit ADAM10. I was unable to increase the concentration of the hydroxamate inhibitor further due to toxicity, so as with ADAM17, optimising a knockdown system in primary human macrophages would be extremely useful to provide more evidence to support these conclusions. It would also be interesting to test the involvement of other metalloproteases, such as MT1-MMP and ADAM12, which have been shown to target LRP1 in HT1080 cells (Selvais et al., 2011). The data also suggests that a cysteine protease could potentially be involved, although so far no cysteine protease has been reported to target

LRP1 in any cell type. Unfortunately, due to time constraints, I was unable to investigate this further.

The role of ADAM10 in shedding of LRP1 adds a further level of complexity to the model previously proposed by Schubert *et al.* (2019). It explains how the basal level of LRP1 shedding I observed is achieved without stimulation of ADAM17. This constitutive level of shedding would mean that steady-state levels of TIMP-3 are maintained on the cell surface. According to the Schubert model, when TNF release is initiated by LPS stimulation, ADAM17 is activated, and TIMP-3 is endocytosed by LRP1. LRP1 shedding is then stimulated more slowly or at a later time point, resulting in build-up of TIMP-3 on the cell surface and inhibition of ADAM17-mediated TNF release.

The results in this thesis show that the LPS-stimulated LRP1 shedding, is potentially mediated by ADAM10. This role of ADAM10 could potentially explain how LPS stimulation results in both the initial TNF release and the later shedding of LRP1 and termination of TNF release. As I mentioned, ADAM10 stimulation by LPS is downstream of other signalling events which would delay its activation in response to LPS.

Another aspect of this model I would have liked to investigate in more detail, if I had more time, would be the effect of inhibiting or increasing LRP1 shedding on TNF release. When stimulated with LPS, primary human macrophages rapidly released TNF, but the rate of release had started to decrease by 6 h and TNF release plateaued by 12 h. If LRP1 shedding was inhibited, I would expect the rate of TNF release to be maintained for longer and to reach a plateau at a higher level. If ADAM10 was inhibited and LRP1 shedding reduced, then endocytosis of TIMP-3 by LRP1 would increase and hence I would predict there to be an increased, or prolonged activation of ADAM17 and a higher TNF release. I was only able to test this at the one time point (6 h) and TNF release was not altered either by inhibition with GI 254023X or by stimulation with ionomycin. I think that this is most likely a time point issue and that testing the effects of these treatments at several time points over 24 h would give a clearer indication of effects on TNF release.

6.3.2 Potential role of tetraspanins in regulating the ADAM10-mediated shedding of LRP1 from human macrophages

Once I had investigated which protease was responsible for shedding LRP1 from human macrophages, one of my aims was also to investigate how that shedding may be regulated. Unfortunately, due to time constraints, I was unable to make much progress towards this aim although I briefly tested how the expression of the TspanC8s changed in response to LPS stimulation.

The TspanC8 family of tetraspanins are a subgroup of tetraspanins which have been shown to regulate the trafficking, localisation, and substrate selectivity of ADAM10 (Dornier et al., 2012; Eschenbrenner et al., 2020; Harrison et al., 2021; Jouannet et al., 2016). I hypothesised that the TspanC8s may regulate the ability of ADAM10 to shed LRP1 as they do with other ADAM10 substrates such as the platelet-activating collagen receptor GPVI (Koo et al., 2022), neuronal N-cadherin (Jouannet et al., 2016), and Notch (Dornier et al., 2012; Ruiz-García et al., 2016). The ability of ADAM10 to shed LRP1 could be dependent on which TspanC8 it interacts with. This may also explain why ADAM10 sheds LRP1 in some cell types but not others, as different cell types express different repertoires of TspanC8s (Matthews et al., 2018).

From the albeit limited data I collected, I hypothesised that the changes in TspanC8 expression in response to LPS stimulation that I observed might indicate that the primary TspanC8 that interacts with ADAM10 changes in response to LPS stimulation. As *TSPAN15* was downregulated and *TSPAN33* upregulated in response to LPS, I speculate that ADAM10/TSPAN33 complex may be better at cleaving LRP1 than the ADAM10/TSPAN15 complex. TspanC8 switching could thus be a mechanism by which the ADAM10-mediated shedding of LRP1 is upregulated in response to LPS. Increased expression of *TSPAN33* in response to LPS has previously been shown to increase ADAM10 activity in macrophages (Ruiz-García et al., 2016). In this study, the LPS-stimulated ADAM10/TSPAN33 complex was shown to cleave Notch, a well known ADAM10 substrate, thereby increasing Notch signalling, which co-operates with TLR signalling to promote macrophage activation. While Ruiz-Garcia *et al.* (2016) focused on Notch, LPS-induced expression of *TSPAN33* may also promote cleavage of other ADAM10 substrates, such as LRP1. If this were the case, TSPAN33 would contribute to the activation of inflammation via Notch signalling, but also at the same time increase the ADAM10-mediated shedding of LRP1, to inhibit the release of TNF and regulate inflammation. This would provide another mechanism by which Notch and TLR signalling interact to reciprocally regulate their respective pathways (Hu et al., 2008).

However, I had insufficient time to test this hypothesis. As knockdown of *ADAM17* and *ADAM10* in primary macrophages proved difficult to optimise, knockdown of the TspanC8s would probably not be an appropriate method of investigate this and attempting to overexpress *TSPAN33* in primary macrophages might also be technically challenging. This could be solved by using CRISPR/Cas9 to either knockout or overexpress the TspanC8s in human iPSCs before differentiating to macrophages and measuring LRP1 shedding by western blotting. It would also be possible to use replication-deficient adenovirus as a vector to overexpress the TspanC8s in primary human macrophages as has previously been done for other proteins (Campbell et al., 2004).

6.3.3 The role of ADAM10-mediated LRP1 shedding in inflammation

The regulated resolution of TNF release is a crucial step in the healthy inflammatory response to infection and injury. Prolonged TNF release is associated with many chronic inflammatory conditions such as rheumatoid arthritis (Feldmann et al., 1995; Radner & Aletaha, 2015) and Crohn's disease (van Dullemen et al., 1995).

I propose that ADAM10-mediated shedding of LRP1 from macrophages can contribute to regulation of the inflammatory response (Figure 6.1). This process can add to the multiple mechanisms that prevent constitutive TNF release, and importantly also inhibit LPS-stimulated TNF release to help prevent a prolonged inflammatory response.

Previous studies show that the role of LRP1 in inflammation is a complex one. Some pro-inflammatory effects have been observed, with, for example, improved atherosclerotic regression and resolution of inflammation in LRP1-deficient mice (Mueller et al., 2018). However, a greater number of studies have indicated that LRP1 has an anti-inflammatory role, with deletion of LRP1 in macrophages increasing atherogenesis in mice (Overton et al., 2007), and LRP1-deficient murine macrophages being more sensitive to pro-inflammatory stimuli (May et al., 2013) and having increased activation of the NF-κB pathway (Gaultier et al., 2008). These studies fit with my data and the model proposed by Schubert *et al.* (2019), in which LRP1 contributes towards resolution of the inflammatory response, since its shedding leads to TIMP-3 accumulation and reduced TNF release. My results additionally predict that defects in ADAM10 function are likely to switch the effect of LRP1 from an anti-inflammatory to a pro-inflammatory one, where LRP1 continues to endocytose TIMP-3 and so promote TNF release.

Much of the research on LRP1 has been done using mice and murine macrophages, and I have shown that human macrophages have different mechanisms of LRP1 shedding. However, it has been observed in both mice and humans that there is an increased level of shed LRP1 in chronic inflammatory conditions (Gorovoy et al., 2010; Llorente-Cortés et al., 2004; Luoma et al., 1994; Quinn et al., 1997). This suggests that increasing the shedding of LRP1 in response to inflammatory stimuli is not enough on its own to limit chronic inflammation and rather that LRP1 shedding is a method of fine-tuning the inflammatory response.

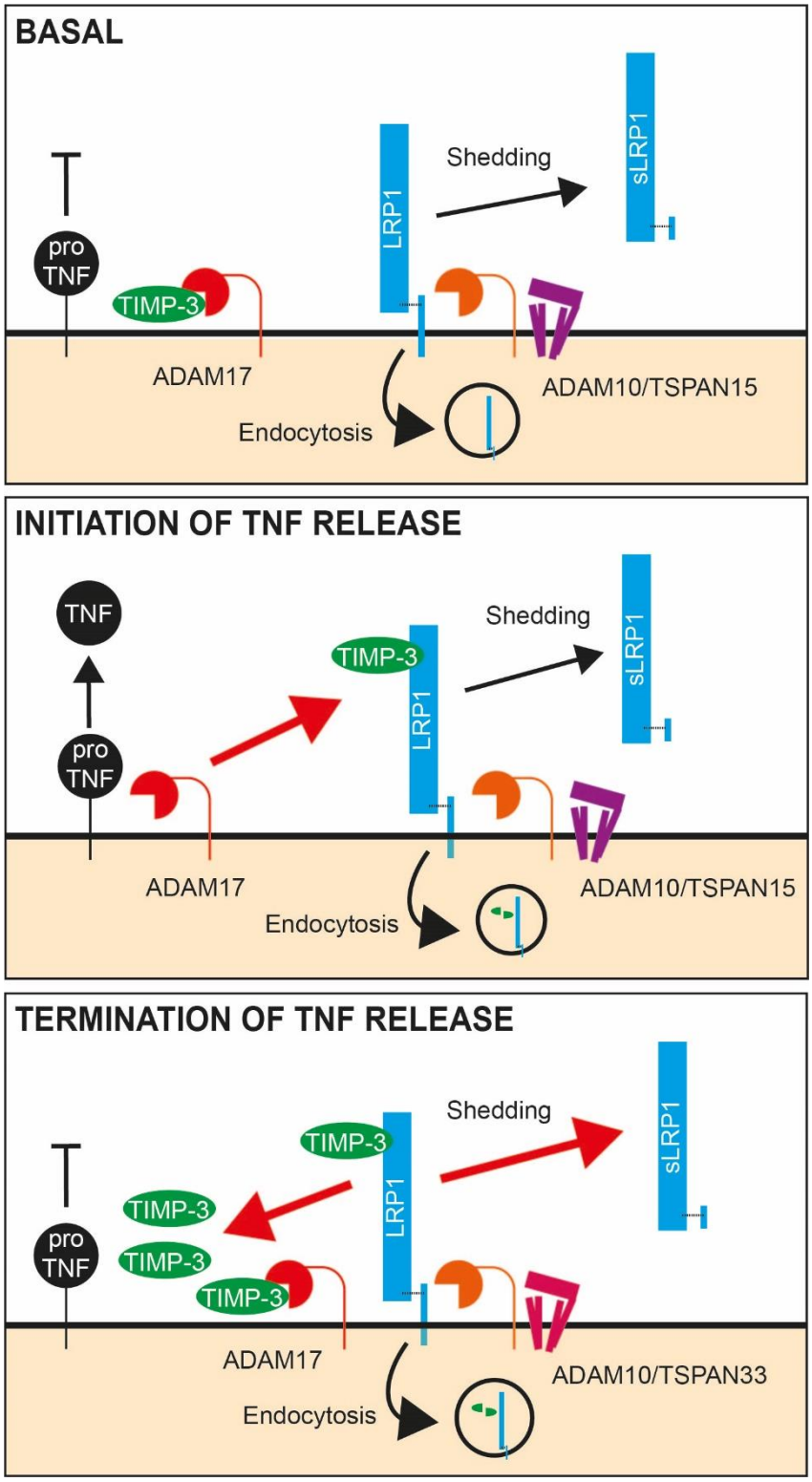

Figure 6.1 Proposed model of ADAM10-mediated shedding of LRP1 and how it regulates the inflammatory response.

In a basal, unstimulated state, there is a balance between ADAM10 constitutively shedding LRP1 and LRP1 endocytosis. Inactive ADAM17 is in a complex with TIMP-3. Upon stimulation with pro-inflammatory mediators such as LPS, ADAM17 is rapidly activated, causing a confirmational change which leads to TIMP-3 dissociating from ADAM17 and being endocytosed by LRP1, removing the inhibitor from the cell surface. At this stage the

rates of shedding and endocytosis of LRP1 have not changed. Later, the LPS stimulation increases the rate of ADAM10-mediated LRP1 shedding, potentially by inducing *TSPAN33* expression and a switch in which Tspan is bound to ADAM10. This results in a build up of TIMP-3 on the cell surface to a level which is sufficient to inhibit the release of TNF by ADAM17, hence contributing to the resolution of inflammation.

6.3.4 Therapeutic targeting of ADAM10-mediated LRP1 shedding

One of the major strategies for treating chronic inflammatory conditions such as rheumatoid arthritis is the use of anti-TNF therapies (Feldmann, 2002). As TNF is the first pro-inflammatory cytokine to be produced following initiation of the inflammatory response (Feldmann et al., 1999; Fong et al., 1989), it is generally considered to be the primary inflammatory mediator and so in an appealing target for therapeutic interventions. Most of these therapies take the form of monoclonal antibodies targeting TNF and these have been approved for the treatment of several chronic inflammatory diseases. However, these therapies are most effective when administered early in the development of disease (Goekoop-Ruitermann et al., 2008) and so for patients whose disease is well established or not detected early enough, these therapies can have limited use (Monaco et al., 2015). Even when treated early, 30-40% of patients are unresponsive and 28% develop anti-drug antibodies within three years of treatment (Rubbert-Roth et al., 2019). It is common practice to switch to a different TNF inhibitor, but these will often have the same mechanism of action and so will have the same problems. Therapies with a different mode of action are therefore necessary for use instead of, or in combination with, established anti-TNF therapies.

Targeting the pathway resulting in the release of TNF from macrophages could provide a strategy for this. ADAM17 has been a target of interest for the development of therapeutics to treat chronic inflammatory diseases since it was discovered to shed TNF (Black et al., 1997). This has been attempted using a range of methods including small-molecule (Moss & Minond, 2017) or hydroxamate-based inhibitors (Hirata et al., 2017), synthetic TIMPs which are resistant to LRP1-mediated endocytosis (Doherty et al., 2016), and blocking antibodies (Rios-Doria et al., 2015). However, one of the major issues with this strategy is the number of biological processes that ADAM17 is involved in, and its ubiquitous expression in human cells. Systemic inhibition of ADAM17 is therefore likely to result in side effects. The similarity between the ADAM17 catalytic site and the catalytic site of other metalloproteases also means ADAM17 inhibitors often cross react with other metalloproteases, causing additional off-target effects (Calligaris et al., 2021).

Development of novel therapeutics in this area has thus largely been focused on directly inhibiting TNF or on trying to prevent its release by inhibiting ADAM17. Further

understanding of the pathway that results in ADAM17-mediated TNF release and its regulation could provide more potential targets, which could help to target therapeutics specifically to macrophages and prevent off-target side effects.

The data presented here identify ADAM10 as a different potential target for therapeutic intervention, and since ADAM17 is the primary LRP1 sheddase in other human cell types, this could allow for the specific targeting of LRP1 shedding in macrophages. However, the number of primary human cell types in which this pathway has been investigated is small and so much more work would be needed to validate this approach. If successful, this could enable development of alternative treatments for chronic inflammatory conditions instead of or in combination with, direct inhibition of inflammatory cytokines. Promoting the normal resolution of the inflammatory response by stimulating the shedding of LRP1 from macrophages in combination with anti-TNF therapies, could prove more effective at treating chronic inflammatory diseases than use of anti-TNF alone. For this to be successful, further research is needed to understand the role and regulation of ADAM10 in other cell types. An additional hurdle is that there are currently no approved therapies directly activating ADAM10.

One method which could be used to modulate ADAM10 activity would be to target tetraspanins. There has been little research so far into how to use tetraspanins as a therapeutic target (Harrison et al., 2021; Robert et al., 2021). The model proposed by Matthews *et al.* (2018) in which ADAM10 is considered to be six different enzymes depending on which TspanC8 it interacts with, would mean that it is possible to target an intervention to be cell-specific or substrate-specific, thereby reducing off-target side effects. Should the role of tetraspanins in the ADAM10-mediated shedding of LRP1 I have proposed be correct, this would be an interesting avenue for further research. Promoting TSPAN33 expression or interaction with ADAM10 may increase the ability of ADAM10 to shed LRP1 while preventing any effects on other ADAM10 functions such as activating Notch signalling which is mediated by TSPAN5 and TSPAN14 (Jouannet et al., 2016).

6.4 Future directions

A useful addition to this project would be the use of flow cytometry to not only confirm the validation of macrophage polarisation by measuring the cell surface levels of various markers, but also to measure the purity of the resulting cells following isolation and differentiation. Even after the two-step density centrifugation and M-CSF differentiation, the resultant population of cells will probably not be completely pure for monocyte derived macrophages. Knowing the purity level and the heterogeneity of the population, would be useful and could go some way to explain the high levels of variation between samples.

One key area for continuing this research would be the further optimisation of a siRNA transfection protocol for the knocking down of genes of interest in the primary human macrophages. This would be necessary to hopefully provide more compelling evidence for the role of ADAM10 in the shedding of LRP1 and to further rule out ADAM17. Should a reliable method of knockdown be optimised, it would then be straightforward to investigate the involvement of other metalloproteases. Whether this optimisation could be achieved using electroporation, or if an alternate method of transfection such as a lipid-based technique like Lipofectamine, would result in more reliable and efficient knockdown would also be important to test.

Optimisation of a knockdown method would also be important for one of the key areas of potential future study, that of the role which tetraspanins play in the ADAM10-mediated shedding of LRP1. Due to time constraints, I was only able to present extremely limited data as to how the expression of the TspanC8s in primary macrophages changes in response to LPS stimulation. Much more work would be necessary to firstly investigate whether any of the TspanC8s play any role in ADAM10-mediated LRP1 shedding, and secondly which tetraspanin(s) are involved. A well-established method for investigating the role of tetraspanins in regard to ADAM10 shedding, is to use knockouts of individual tetraspanins followed by combinations of knockouts. If attempts to optimise a knockdown are unsuccessful, another method of investigating this question could be to look at the localisation of ADAM10 and the TspanC8s at the cell membrane in response to LPS stimulation. The cell surface levels of the different tetraspanins could be measured using flow cytometry at rest and when stimulated with LPS. It would be important to see if the cell surface levels correlated with the changes I observed in gene expression, especially with proteins whose function is involved with trafficking and maturation. ADAM10, LRP1, and a TspanC8 could be also targeted with specific antibodies and then stained to show the location of the individual proteins on the cell surface. This could determine whether ADAM10 colocalises with a TspanC8 at the cell surface and whether that changes in response to LPS stimulation.

Another interesting area for potential future study is investigating the consequences of altering ADAM10-mediated LRP1 shedding for the inflammatory function of the macrophage. Shedding could be inhibited with GI 254023X or induced using ionomycin, and then the TNF release measured with an ELISA. Due to time constraints, I was only able to do this at one time point and observed no effect on TNF release so further work would be required. If my hypothesised model is correct then following inhibition of LRP1 shedding, I would expect the rate of TNF release to be maintained for longer and to reach a plateau at a higher level. It would also be interesting to look at if the polarisation state of the macrophages changed if LRP1 shedding was induced or inhibited. This could be done by

using RT-qPCR to measure the expression of certain markers of polarisation, or by flow cytometry to quantify the surface levels of various markers for polarisation.

A slightly different direction for future work could be to investigate this ADAM10-mediated shedding of LRP1 in a more clinical setting. If blood samples could be obtained from patients with a chronic inflammatory condition, such as RA, as well as measuring the levels of sLRP1 in the serum, one could isolate the macrophages and measure the cell surface levels of LRP1, ADAM10, and TspanC8s using flow cytometry compared with healthy controls. This could give an idea of whether this mechanism of LRP1 shedding is involved in the pathology of chronic inflammation, and maybe suggest further avenues of investigation for the development of therapies to target ADAM10.

As the only cells I investigated in this project was macrophages isolated and differentiated from peripheral blood, it might be another interesting avenue of investigation to look at whether different tissue resident macrophage populations have different mechanisms for regulating LRP1 shedding.

6.5 Conclusions

I have demonstrated that while human monocytic cell lines THP-1 and U937 are useful model systems for some macrophage functions, they are not appropriate for the investigation of LRP1 shedding. Potentially a cell line model for this could be developed by further optimisation of western blotting or flow cytometry or transfecting THP-1 cells with CD14 to improve their LPS sensitivity. Instead, I optimised western blotting to analyse LPS-stimulated shedding of LRP1 from primary human monocyte-derived macrophages, and found this to be an appropriate in vitro model for investigating how LRP1 shedding in these cells is regulated during inflammation.

I found that in vitro polarisation of primary human monocyte-derived macrophages had no effect on LRP1 shedding at the time points I measured. However, further work is needed to confirm this.

The results presented here provide evidence that unlike murine macrophages and other human cell types previously tested (Gorovoy et al., 2010; Liu et al., 2009), human macrophages do not utilise ADAM17 for either constitutive or LPS-stimulated LRP1 shedding. Instead, the metalloprotease ADAM10 plays a key role in LRP1 shedding, both in the basal and LPS-stimulated state. ADAM10 inhibition did not completely inhibit LRP1 shedding, so it is likely that other protease(s) are also involved in this process.

How the shedding of LRP1 by ADAM10 is regulated in response to pro-inflammatory stimuli is an important area for future research. I have proposed a model in which the LPS-stimulated increase in shedding is mediated by switching which member of the TspanC8 family of tetraspanins interacts with ADAM10. This would be an interesting avenue for further research and potentially open a new avenue for therapeutic targeting of TNF release.

This research provides evidence for the role of ADAM10 in inhibition of TNF release from human macrophages and so in resolution of the inflammatory response. It adds to previous research on how LRP1 controls cell surface levels of TIMP-3 to regulate ADAM17 activity (Schubert et al., 2019), further improving our understanding of how macrophages regulate the inflammatory response to prevent chronic inflammation. The more that is understood about how this pathway functions and is regulated, the better our ability to target it with therapeutic interventions will be, and hopefully, this will lead to new methods of treating of chronic inflammatory diseases.

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