TREM-2 Expression and Regulation in Inflammation and Ischaemia

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II) Abstract

Triggering receptor expressed on myeloid cells – 2 (TREM-2) is a receptor expressed mainly in myeloid cells. TREM-2 is involved in the resolution of inflammation through dampening Toll-like receptor (TLR) induced pro-inflammatory cytokine secretion and phagocytic functions. It is increased in inflammatory conditions including rheumatoid arthritis and stroke and also in wound healing. However, little is known about how its expression is regulated. This study analysed the expression of TREM-2 in various myeloid and non-myeloid cell types and investigated the regulation of TREM-2 expression in myeloid cells. TREM-2 was expressed in the Golgi apparatus of microglial cells. Several non-myeloid cell types also expressed TREM-2, including bronchial epithelial cells where it was located on cilia in healthy and diseased lung tissues. In THP-1 cells, the anti-inflammatory cytokines interleukin-4 and transforming growth factor-β1 (TGF-β1) induced TREM-2 expression through phosphoinositide 3-kinase (PI3K) and PI3K/ p38 MAP kinase signalling pathways respectively. TGF-β1-induced TREM-2 also required extracellular signal-regulated kinase 1/2 post-translationally for protein expression. Interestingly, TREM-2 was required for TGF-β1-induced matrix metalloproteinase (MMP)-1 expression, the most characterised MMP in wound healing, suggesting that TREM-2 may be required for the beneficial effects of TGF-β1 on wound healing by regulating MMP-1. An *in vitro* model of ischemia in stroke was then established to study the mechanisms of TREM-2 regulation in stroke. Oxygen glucose deprivation (OGD) had no direct effect on TREM-2 in N9 microglial cells. However, co-culture with healthy neurons reduced microglial TREM-2 expression, which was abolished in co-culture with OGD neurons, suggesting that in the healthy brain, microglial TREM-2 expression is suppressed by neurons, and this suppression is lost during ischaemia, increasing TREM-2 expression. In conclusion, this study characterised TREM-2 expression in non-myeloid cells and identified novel mediators and signalling pathways that regulate TREM-2 expression, which may be responsible for TREM-2 overexpression in inflammatory and ischemic conditions.

III) List of Contents

IV) Published Abstracts and Poster presentations

Bradley, J.M., Squirrell, F., Sexton D.W. and O'Connell M.A. (2013). '**28: Mechanisms of triggering receptor expressed on myeloid cells-2 (TREM-2) regulation by transforming growth factor-β1 and interleukin-4; a new role for TREM-2 in matrix metalloproteinase regulation**.' *Cytokine* 63(3): 249-250. Cytokines: From Molecular Mechanisms to Human Disease, 29th September – 3rd October 2013 (San Francisco, UK).

Bradley, J.M., Squirrell, F., Sexton, D.W., O'Connell, M.A. (2012) '**Mechanisms of TGF-β1 and IL-4-induced TREM-2 expression in monocytes'** *Immunology* 137: 185-772. European Congress of Immunology 5th-8th September 2012 (Glasgow, UK).

Bradley, J.M., Norton, R., Sexton, D.W., O'Connell, M.A. (2011) '**TREM-2: expression in non-myeloid cell types and regulation by inflammatory mediators'.** *Immunology* 135: 52-212. BSI congress 2011, 5th-8th December 2011 (Liverpool, UK).

V) Oral Presentations

Bradley, J.M., Squirrell, F., Sexton D.W. and O'Connell M.A. **'Regulation and functional effects of TGF-β1 induced TREM-2 expression in inflammatory disease'.** Pharmacy Research Day: 27th June 2013 (University of East Anglia, UK).

Bradley, J.M., Squirrell, F., Sexton D.W. and O'Connell M.A. **'Regulation and function of TREM-2 by TGF-β1 and IL-4 in human monocytes'**. Early Careers Inflammation and Immunology Symposium 26th March 2013

Bradley, J.M., Norton, R., Sexton, D.W., O'Connell, M.A. **Expression and regulation of TREM-2 in innate immune cells.** Early Careers Inflammation and Immunology Symposium 6th June 2011.

VI) Papers

Bradley, J.M., Squirrell, F., Sexton, D.W., O'Connell, M.A. (2013) **'TGF-β1 and IL-4 increase TREM-2 expression in monocytes via MAPK and PI3K signalling pathways'.** – In preparation, for submission to the *Journal of Immunology*.

VII) List of Tables

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IX) Abbreviations

AD	Alzheimer's Disease
AIF-1	Allograft inflammatory factor 1
AP-1	Activating protein-1
APS	Ammonium persulfate
Arg-1	Arginase 1
ATF2	Activating factor 2
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCR	Chemokine (C-C motif) receptor
cDNA	Complimentary DNA
C/EBPα	CCAAT/enhancer binding protein alpha
CFSE	Carboxyfluorescein succinimidyl ester
CHO	Chinese Hamster Ovary
Cdc-42	Cell division cycle 42
CNS	Central Nervous System
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CSF	Cerebrospinal fluid
CT	Comparative threshold
CX3CL	Chemokine (C-X3-C motif) ligand
CXCL	Chemokine (C-X-C motif) ligand
DAMP	Damage associated molecular patterns
DAP12	TYROBP/ KARAP (TYRO protein tyrosine kinase binding protein)
DC-STAMP	Dendritic cell specific transmembrane protein
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOK3	Docking protein 3
dNTPs	Deoxynucleotide Triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase

FCS	Fetal calf serum
GABA	Gamma-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA1	GATA binding protein 1
GM-CSF	Granulocyte macrophage colony stimulating factor
Grb2	Growth factor receptor bound protein 2
HBSS	Hanks buffered salt solution
HCL	Hydrochloric acid
HORC	Human organotypic retinal culture
HSC	hematopoietic stem cell
HSP	Heat shock protein
ICAM	Intracellular adhesion molecule
IFN	Interferon
IKK	IκB kinase
IL	Interleukin
IL-1RA	Interleukin 1 receptor antagonist
IMDM	Iscove's modified Dulbecco's media
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated *kinase 1 and 4*
IRF-4	Interferon regulatory factor 4
IRS	Insulin receptor substrate
ITAM	Immune tyrosine receptor based activation motif
IκB	Inhibitor κB
JAK	Janus kinase
JMJD3	Histone Demethylase Jumonji D3
JNK	c-Jun N-terminal kinase 1
KD	Knockdown
KO	Knockout
LAB	Linker for activation of B cells (NTAL or LAT2)
LDS	Lithium dodecyl sulfate
LPS	Lipopolysaccharide
LT	Leukotriene
MAPK	Mitogen activated protein kinase
MCAo	Middle Cerebral Artery occlusion
MCL-1	Myeloid leukaemia cell differentiation protein
M-CSF	Macrophage colony stimulating factor

MDL-1	Myeloid DAP12-associating lectin-1
MDPs	Macrophage dendritic cell progenitors
MEK1	Mitogen-activated protein kinase 1
MFG-E8	Milk fat globule-EGF factor 8 protein
MHC	Major histocompatibility complex
MITF	Microphthalmia-associated transcription factor
MMP	Matrix metalloproteinase
MR	Mannose receptor
MS	Multiple sclerosis
MTS	3-(4,5-dimethylthiazol-2-yl)- 5-(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium
MyD88	Myeloid differentiation factor 88
MyoD	Myogenic differentiation 1
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NFATc	Nuclear factor of activated T-cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve Growth Factor
NHD	Nasu-Hakola Disease
NK	Natural killer
NLR	Nod-like receptor
NO	Nitric oxide
OGD	Oxygen glucose deprivation
OCT	Optimum cutting temperature
oxLDL	Oxidised low density lipoproteins
PA-1	Plexin A1
Pam3Cys	(*S*)-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser(*S*)-Lys_4-OH, trihydrochloride
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
Pen/Strep	100 U/mL penicillin and 100 µg/mL streptomycin (pen/strep)
PFA	Paraformaldehyde
PG	Prostaglandin
PGN	Prostaglandin
PGN	Peptidoglycan
PI3K	Phosphoinositide 3-kinase

PLC	Phospholipase C
PLOSL	Polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy
PMA	Phorbol-12 myristate 13-acetate
PMNs	Polymorphonuclear cells
PPAR-γ	Peroxisome proliferator activated receptor gamma
PRR	Pattern Recognition Receptors
PS	Phosphatidylserine
PU.1	SPI1; spleen focus forming virus (SFFV) proviral integration oncogene
PVDF	Polyvinylidene difluoride
Pyk2	Proline rich tyrosine kinase 2
qRT-PCR	Quantitative real time polymerase chain reaction
RANK	Receptor Activator of NFκB
Rho	Ras homolog family member
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RUNX1	Runt-related transcription factor 1
SAECs	Human Small Airway Epithelial Cells
SDS	Sodium dodecyl sulfate
SDF-1	Stromal cell-derived factor-1
Sema6D	Semaphorin
SH2	Src homology 2
Shc	Src homology 2 domain containing
shRNA	Small hairpin RNA
siRNA	Small interfering ribonucleic acid
SHIP	SH2-containing inositol phosphatase
SIRP β	Signal regulatory peptide beta
SMAD	Mothers against decapentaplegic homolog
SNPs	Single nucleotide polymorphisms
STAT	Signal-transducer and activator of transcription protein
sTREM-2	Soluble TREM-2
Syk	Spleen tyrosine kinase
TAB	TAK1-binding protein
TAK	TGF-beta-associated kinase

TEMED	Tetramethylethylenediamine
TGF-β1	Transforming growth factor beta 1
TGF-β1R	Transforming growth factor beta receptor I
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll like receptor
TLT	TREM like transcript
TNF-α	Tumour necrosis factor alpha
TRAF6	TNF receptor-associated factor 6
TREM	Triggering receptor expressed on myeloid cells
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UDP	Uridine diphosphate
VCAM	Vascular adhesion molecule
VIP	Vasoactive intestinal peptide
β-COP	Beta-coatomer-protein

1. Introduction and Aims

1.1. Inflammation and the Immune Response

Inflammation is the reaction of the body to an immune response initiated by external or internal stimuli for example pathogenic organisms, physical damage or oxidative stress (Figure 1.1). Ischaemia is one of the major inducers of oxidative stress and therefore ischaemic conditions such as cardiovascular disease, age related macular degeneration and ischaemic colitis are often associated with inflammation [1-3]. The classical signs of acute inflammation include redness, swelling, pain and heat.

1.1.1. Initiation of the Inflammatory Response

Macrophages and dendritic cells resident in the tissues or monocytes in the blood identify the stimulus and initiate an immune response. Ischaemia initiates this response through production of reactive oxygen species (ROS) which cause the release of pro-inflammatory mediators including TNF-α and IL-8, increasing inflammation [4, 5]. The inflammatory response can also be initiated by binding of pattern or damage associated molecular patterns (PAMPS or DAMPS) to pattern recognition receptors (PRRs) [6]. The toll-like receptors (TLRs) are the major class of PRR. PAMP molecules include lipids, DNA and RNA, they are exposed to the host cells by infectious organisms and bind and activate TLRs initiating an intracellular signalling cascade resulting in the release of pro-inflammatory mediators (Figure 1.1) [6]. Lipopolysaccharide (LPS) is a PAMP found on the surface of gram-negative bacteria, this molecule activates TLR4 to initiate the host's immune response against invading bacteria [7]. TLR3 on the other hand, recognises double stranded RNA molecules, in particular those found in viruses, to alert the host of virus infection and initiate the innate immune response [8]. Interestingly, DAMPS produced endogenously including DNA damage induced by ultraviolet radiation [9] or heat shock proteins (HSPs) [10], fibrinogen [11] and hyaluronic acid fragments [12] induced by tissue injury also cause the activation of TLRs activating the inflammatory response. This causes the initiation of processes to repair the injury or cause the clearance of the cause of the DAMPS e.g. dying cells, cell debris or pathogens [13, 14].

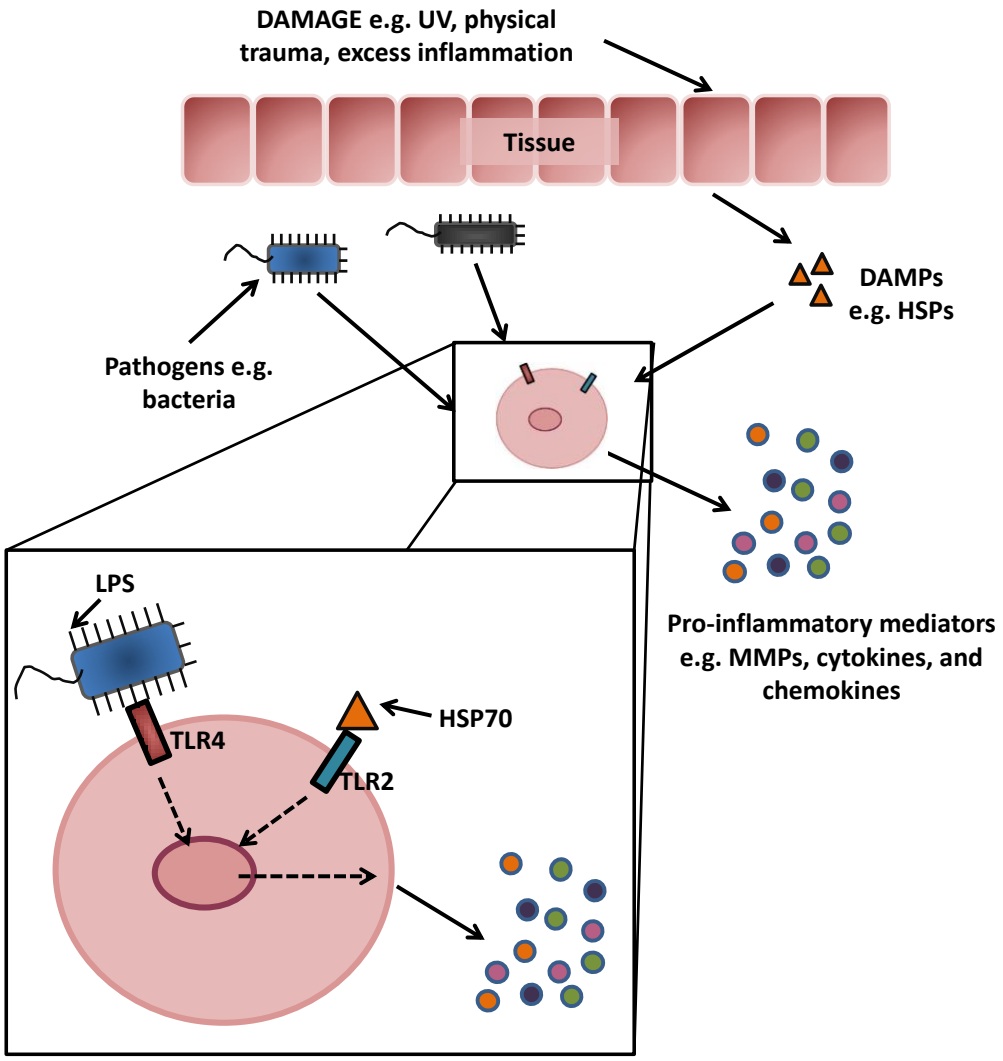

Figure 1.1: DAMPs and PAMPs activate TLR receptors. DAMPs including HSPs [15] and PAMPs including LPS activate TLR receptors resulting in the regulation of pro- and anti-inflammatory mediators including MMPs (matrix metalloproteinases), cytokines and chemokines.

1.1.2. Phase I of the Inflammatory Response

The inflammatory response occurs in two phases. Phase I is the initial inflammatory phase and is important for the killing of invading organisms and recruitment of inflammatory cells to the damaged area [16]. Phase I of the inflammatory response results in increased expression of pro-inflammatory mediators including cytokines [17, 18], adhesion molecules [19], MMPs [20], prostanoids [21] and chemokines [22] (Figure 1.2). Chemokines are small molecules that attract other inflammatory cells including polymorphonuclear cells (PMNs) such as eosinophils and neutrophils, macrophages, monocytes [23], and lymphocytes [24] to the site of infection or injury.

These cells secrete cytokines and chemokines, further increasing the inflammatory response [25]. The interleukin (IL) family are a major class of cytokines increased during inflammation. The key interleukins released during the acute phase of the inflammatory response are IL-1β and IL-6 [26]. Tumour necrosis factor (TNF)-α is also increased during this phase [27, 28]. Together, these cytokines are important for the progression of the innate immune response, initiating signalling pathways that increase activation of immune cells and cause further secretion of pro-inflammatory mediators required for pathogen killing [16, 27].

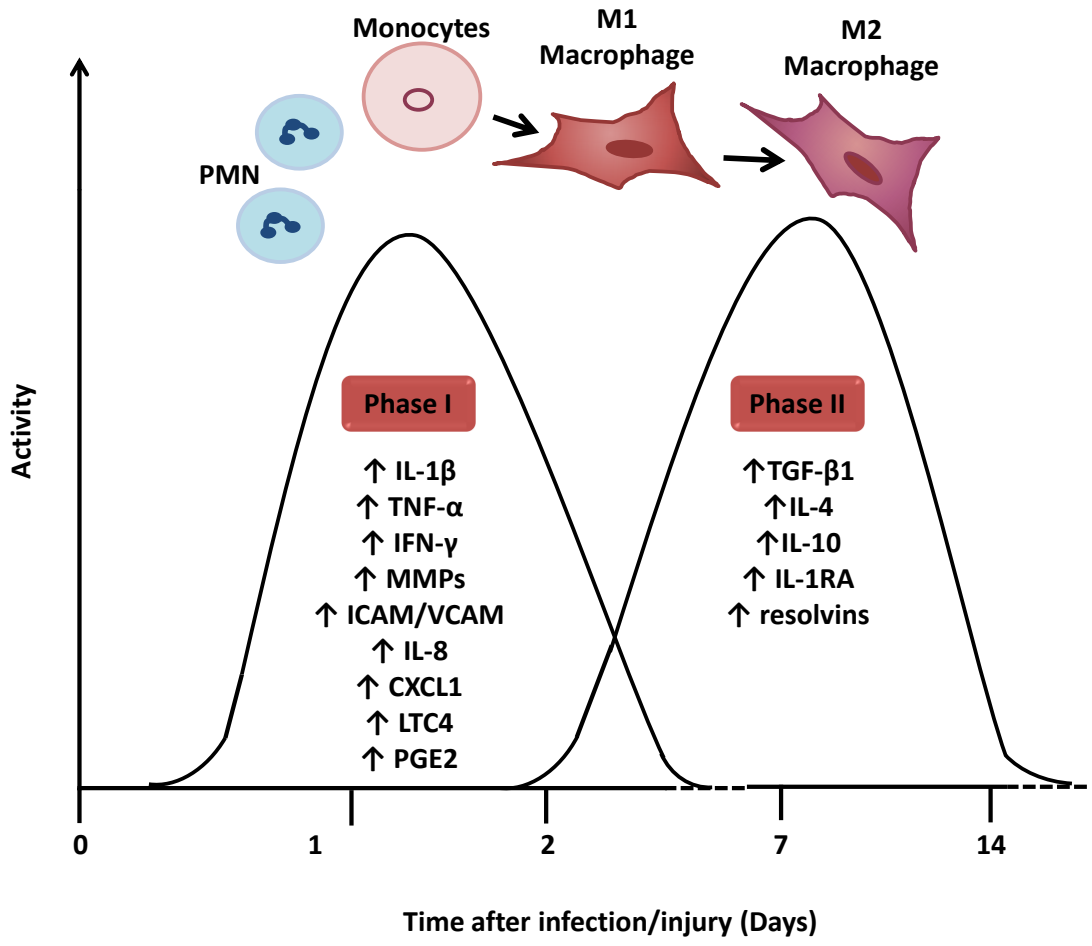

Figure 1.2: Phase I and Phase II of the immune/inflammatory response after infection or injury. The first phase of inflammation (Phase I) is characterised by increases in pro-inflammatory mediators including IL-1β, TNF-α, interferon-γ (IFN-γ), MMPs, intracellular adhesion molecules (ICAMs), vascular cellular adhesion molecules (VCAMs), IL-8, the chemokine chemokine (C-X-C motif) ligand (CXCL)-1, leukotriene C4 (LTC4) and prostaglandin E_2 (PGE_2). The second phase of inflammation (Phase II) usually occurs days to weeks after initiation and is characterised by an increase in mediators including transforming growth factor β1 (TGF-β1), IL-4, IL-10, IL-1 receptor antagonist (IL-1RA) and resolvins.

MMPs secreted during the inflammatory response can cause tissue damage by degrading the extracellular matrix. Tissue damage caused by elevated MMPs is present in many inflammatory diseases including asthma and rheumatoid arthritis [29, 30]. The role of MMPs in inflammation is complex. MMPs have both pro- and anti-inflammatory effects. MMPs increase the inflammatory response through cleavage of pro-inflammatory chemokines and cytokines into their active forms. For example, MMP-2 cleaves pro-IL-1β into IL-1β which activates this cytokine [31]. They can also reduce inflammation by degrading mature forms of cytokines reducing their activity e.g. MMP-1 degrades IL-1β [32]. Aside from their role in inflammation, MMPs are important in tissue repair and regeneration. For example, MMP-1 is important in wound healing, it promotes angiogenesis and epithelial migration increasing the healing process [33-35]. This highlights the importance of the regulation of MMPs for the maintenance of tissue homeostasis and in inflammation and injury.

Adhesion molecules including VCAM-1 and ICAM-1 are also increased during the acute phase of the inflammatory response [19]. These molecules are found on the surface of both immune and non-immune cells and increase immune cell binding and translocation across epithelial and endothelial barriers [36]. High levels of these adhesion molecules increases immune cell influx to the site of injury, increasing inflammation.

Prostanoids are also increased in Phase I of the inflammatory response [37]. Prostanoids are a group of inflammatory mediators including leukotrienes, thromboxanes and prostaglandins. Leukotrienes such as LTC4, increase inflammatory cell activation and cytokine secretion increasing the local inflammatory response [38]. Prostaglandins are also increased in this phase, particularly PGE_2 [39]. PGE_2 acts on sensory neurons resulting in the sensation of pain following injury and during inflammation [40]. PGE_2 also increases blood flow to the site of injury increasing immune cell influx and therefore inflammation [41]. One of the main anti-inflammatory drugs, aspirin inhibits cyclooxygenase (COX) enzymes that are required for the synthesis of the prostanoids. Inhibition of COX reduces prostanoid synthesis and this is how aspirin reduces inflammation further highlighting the importance of prostanoids in acute inflammation [42].

The differentiation of infiltrating monocytes into macrophages or dendritic cells is another key stage of the acute phase response. The differentiation fate of the cell is governed by the mediators in the surrounding environment (Figure 1.3). For example, the presence of LPS and/or IFN-γ will encourage the differentiation of

monocytes into M1-type macrophages that secrete high levels of pro-inflammatory mediators including IL-1β and TNF-α (Figure 1.3) [43]. The presence of IL-4 in the environment will encourage differentiation into dendritic cells or M2-type macrophages (Figure 1.3) [44]. M1 macrophages are the 'classically activated' macrophages with a more pro-inflammatory phenotype, whereas the M2 type macrophages are 'alternatively activated' and have a more anti-inflammatory phenotype (Figure 1.4) [45, 46]. The M2-type macrophages are associated with the resolution of inflammation through increased clearance of cell debris, suppression of pro-inflammatory cytokine secretion and increased anti-inflammatory cytokine secretion [47, 48]. Once the inflammatory cascade has been initiated, cytokines activate other cells, including endothelial cells [49], eosinophils [50], neutrophils [51] and T- lymphocytes (T-cell) [52], which can induce further cytokine secretion and inflammation (Figure 1.4). There are a number of different types of T-cell, and similarly to macrophages, the mediators in the surrounding environment decide the T-cell fate and the inflammatory mediators produced, these are detailed in Figure 1.4.

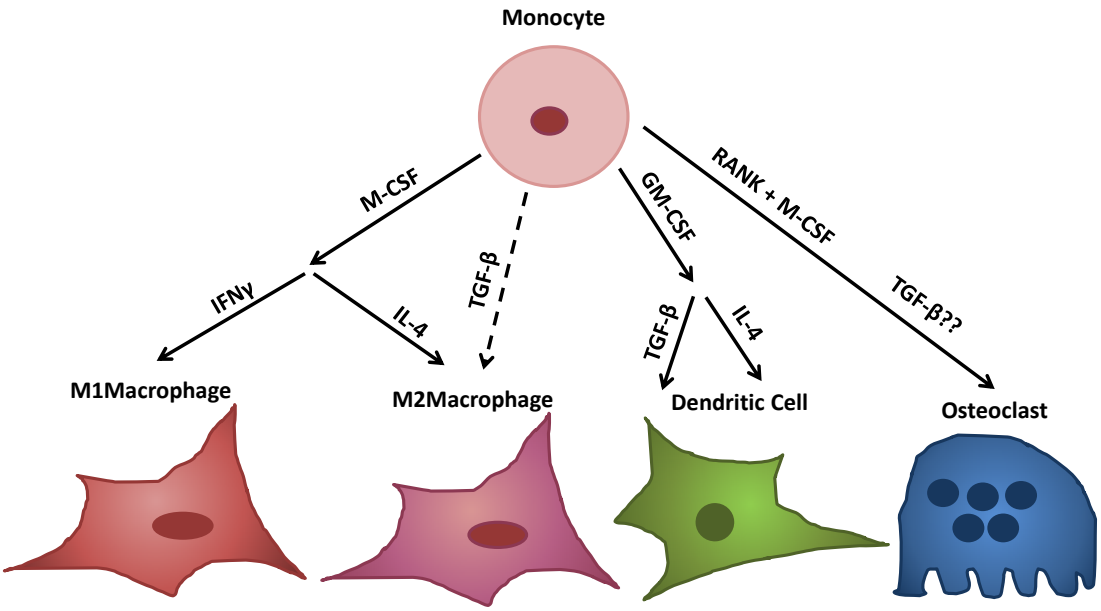

Figure 1.3: Myeloid differentiation. Differentiation of monocytes into other myeloid cell type including macrophages (M1 and M2), dendritic cells and osteoclasts by M-CSF (macrophage colony stimulating factor), IFN-γ, IL-4, GM-CSF (granulocyte M-CSF), RANK (receptor activator of NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells)) and TGF-β.

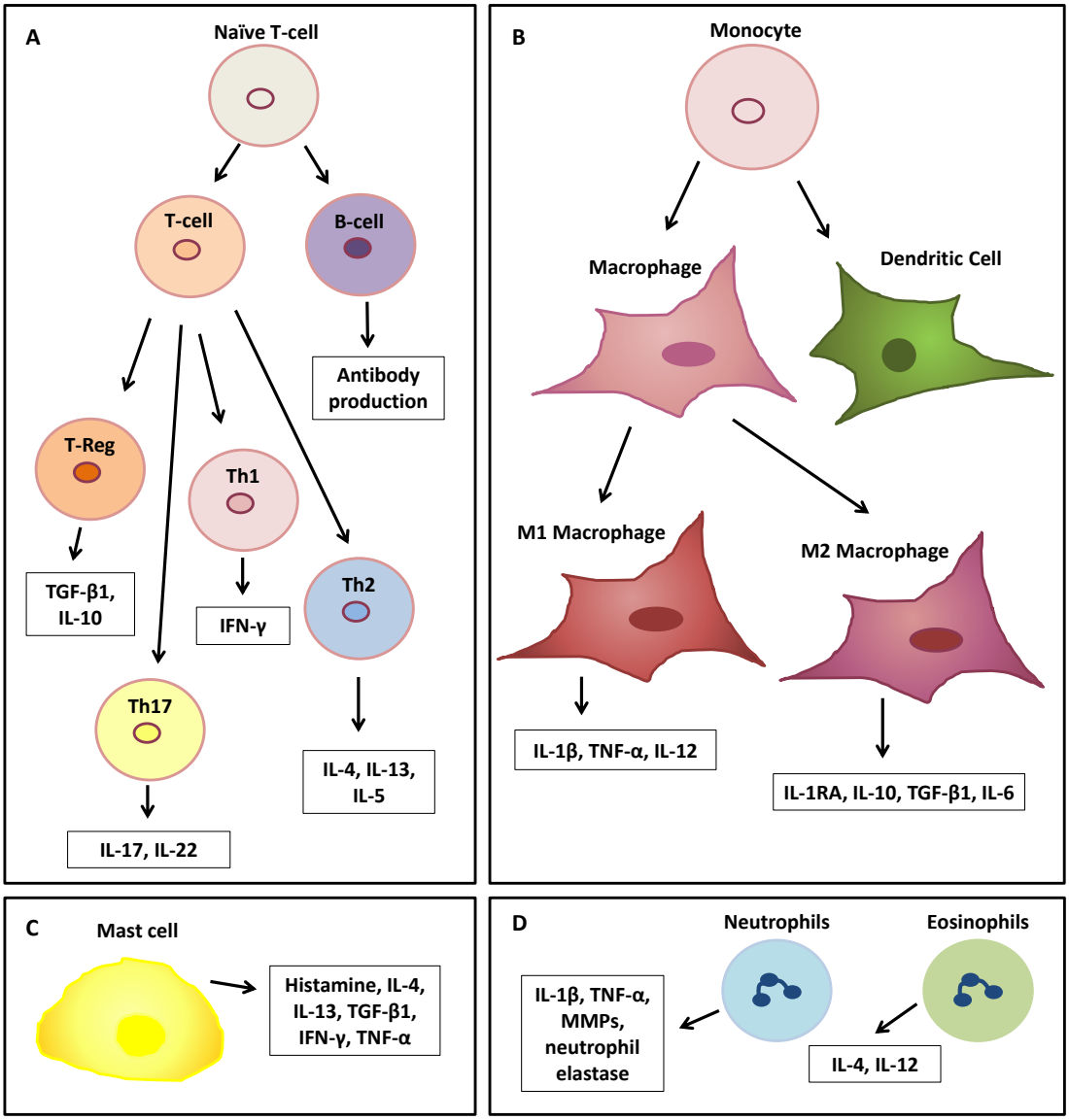

Figure 1.4: Immune cell types with cytokines secreted. (A) T – lymphocyte (T-cell) subsets, (B) Myeloid subsets, (C) mast cells and (D) granulocytes [53-57].

The major signalling pathway required for the production and activation of these pro-inflammatory mediators is the NFκB pathway (Figure 1.5) [58]. Activation of this transcription factor alters gene expression of many components of the inflammatory response including upregulation of pro-inflammatory mediators, including IL-1β, TNF-α, IL-6 and IL-8 [58]. NFκB is activated by a variety of stimuli including TLR activation, ROS [59] and IL-1β and TNF-α receptor activation [6, 60]. Of these signalling pathways, TLR mediated NFκB activation is the most characterised. TLR activation results in the recruitment and activation of a variety of intracellular signalling molecules including myeloid differentiation factor 88 (MyD88), interleukin-1 receptor-associated *kinase* (IRAK) 1 and 4, TNF receptor-associated factor 6

(TRAF6), TGF-beta-associated kinase 1 (TAK1) and TAK1-binding protein (TAB) proteins (Figure 1.5) [61]. This signalling pathway results in the activation of inhibitor κB (IκB) kinase (IKK) which degrades IκB [62]. IκB is the inhibitory molecule that associates with NFκB to prevent its activation [62]. Degradation of this molecule allows NFκB activation and translocation into the nucleus to regulate the expression of many genes required for the inflammatory response [62].

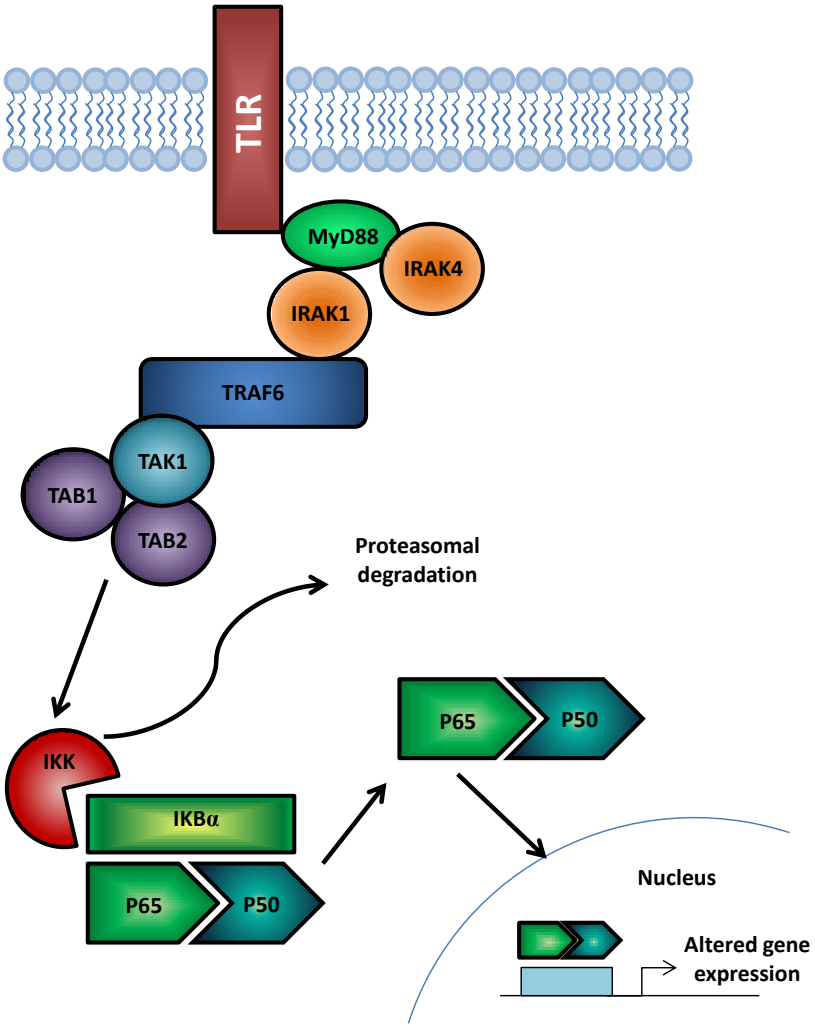

Figure 1.5: NFκB signalling following TLR activation. TLR activation activates the MyD88 signalling pathways though IRAK 1 and 4, TRAF6, TAK1 and TAB1 and 2. TAK1 and TAB 1 and 2 activate IKK phosphorylating IκBα resulting in IκBα proteasomal degradation. This allows NFκB components p65 and p50 to bind to NFκB response elements in key genes altering inflammatory gene expression.

Interestingly, NFκB also upregulates anti-inflammatory cytokines including TGF-β and indirectly IL-10 via cytokines such as TNF-α [63-66]. The release of these anti-inflammatory cytokines is one of the anti-inflammatory mechanisms put in place to

prevent over-activation of the inflammatory response. This inflammatory cascade is important for protection from infection and from tissue damage by killing invading organisms, protecting the injury site from infection (Phase I) and to initiate the repair process and resolution of inflammation (Phase II) [67, 68]. Inflammation is the cause or a symptom of most diseases including atherosclerosis [69], cancer [70], stroke [71], arthritis [72], Alzheimer's disease (AD) [73], asthma [74], diabetes [75] and many more, highlighting the importance of the resolution of inflammation.

1.1.3. Phase II of the Inflammatory Response

Phase II of the inflammatory response initiates processes that dampen down inflammation initiated in the Phase I, to reduce tissue damage caused by inflammation (Figure 1.2). One mechanism is through apoptosis of immune cells including neutrophils and macrophages to reduce immune cell secretion of pro-inflammatory mediators [76, 77]. However, the presence of apoptotic cells can increase inflammation and therefore another important part of this process is phagocytosis [78]. During this phase, apoptotic and necrotic cells and cell debris are cleared up by phagocytes including dendritic cells and macrophages [79]. Clearance of dying cells reduces the inflammation by preventing the secretion of these pro-inflammatory mediators and through the secretion of anti-inflammatory cytokines by the phagocyte including IL-10 [80, 81]. Anti-inflammatory cytokines are released by immune cells to reduce pro-inflammatory mediator production [82, 83]. Another group of anti-inflammatory mediators released during Phase II are the resolvins [84]. Resolvins reduce inflammatory pain by directly blocking pain pathways and reducing pro-inflammatory mediators in the surrounding environment [85, 86]. Also during this phase, the IL-1RA is released to negatively regulate IL-1β signalling. IL-1RA acts as an inhibitor of IL-1β signalling by binding to the IL-1 receptor, blocking the access of IL-1β to its receptor [87]. Other key anti-inflammatory mediators are the thromboxanes which are also increased in Phase II. Thromboxanes increase growth factor secretion, stem cell migration and angiogenesis, reduce inflammation and increase tissue repair [88-90].

1.1.3.1. IL-4 Signalling and Function

Th2 cytokines including IL-4, IL-5 and IL-13 can also be increased during Phase II [91, 92]. These cytokines are secreted primarily by Th2 type T-cells and have been shown to reduce inflammation induced by Th1 type cytokines including IFN-γ and TNF-α [93]. IL-4 and IL-13 can signal via the same receptor and therefore have similar signalling pathways and functions [94]. IL-4 is also produced by mast cells, basophils and eosinophils [95]. IL-4 acts on leukocytes, in particularly monocytes, to reduce proinflammatory cytokine and chemokine production including IL-1β, TNF-α, IL-6 and IL-8 [96, 97]. This suppression of cytokine secretion induced by IL-4, reduces inflammation and reduces symptoms of inflammatory diseases e.g. in an animal model of arthritis [97]. However, in Th2-type inflammation such as allergy, IL-4 is detrimental, increasing inflammation, exacerbating the disease phenotype [98]. IL-4 also plays an important role in the differentiation of monocytes (Figure 1.3). Co-stimulation of IL-4 with either GM-CSF or M-CSF in monocytes induces differentiation into dendritic cells or M2 type macrophages, respectively [99]. The majority of the literature shows that IL-4 acts on monocytes and macrophages to increase differentiation into the M2 type phenotype [46]. However, IL-4 alone can also induce monocyte differentiation into dendritic cells increasing the expression of many surface markers for dendritic cells, along with morphological changes and increased phagocytic activity [100, 101].

In macrophages, IL-4 activates the IL-4 receptor to induce Janus kinase (JAK) phosphorylation. Phosphorylated JAK activates signal transducer and activator of transcription 6 (STAT6), which activates transcription factor peroxisome proliferator activated receptor gamma (PPAR-γ) and upregulates many genes associated with the M2 phenotype including chitinase 3–like 3 , jumonji domain-containing protein 3, interferon regulatory factor-4 and arginase-1 (arg-1) (Figure 1.6) [102-105]. STAT6 is also required for the anti-inflammatory actions of IL-4 and binds to the promoters of various inflammatory mediators to modulate their expression [106]. IL-4 can also signal independently of STAT6 through insulin receptor substrate 1/2 (IRS1/2) resulting in activation of the phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase 1/2 (ERK 1/2) signalling pathways (Figure 1.6) [107].

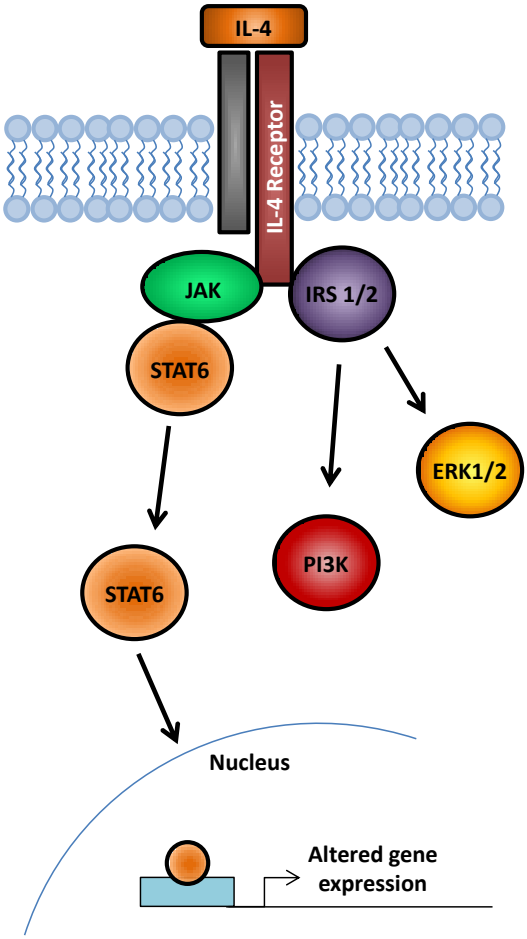

Figure 1.6: IL-4 signalling pathways. IL-4 receptor activation results in JAK STAT6 phosphorylation/activation. IL-4 receptor activation also activates IRS1/2 which in turn activates PI3K and ERK 1/2.

1.1.3.2. TGF-β1 Signalling and Function

TGF-β1 is another important anti-inflammatory mediator increased during Phase II [108]. TGF-β is a growth factor and anti-inflammatory cytokine secreted primarily from regulatory T-cells [109], macrophages [110] and monocytes [111]. TGF-β signals via its receptors, TGF-β1 receptor (TGF-β1R) I and II that are present on many immune cell types including fibroblasts [112] macrophages [113] and monocytes [114]. Activation of these receptors reduces inflammation by suppressing Th1 cell activity [115] and pro-inflammatory cytokine secretion [116] while increasing anti-inflammatory cytokine secretion [117], regulatory T-cell activity [118] and activating signalling to induce tissue repair [119]. In addition knockout (KO) studies have shown that removal of TGF-β1 increases inflammation and reduces survival confirming that TGF-β1 is an important mediator in the resolution of inflammation [120]. However, in monocytes

TGF-β1 can also have pro-inflammatory actions, in particular, recruitment of monocytes to the site of injury [121]. TGF-β1 is also a key inducer of fibrosis by causing excessive secretion of extracellular matrix proteins and MMPs, which can lead to and exacerbate conditions including pulmonary and hepatic fibrosis [122].

As well as being an anti-inflammatory mediator, TGF-β also modulates myeloid cell differentiation (Figure 1.3). TGF-β alone induces partial differentiation of promyelocytic cells into monocytes and macrophages [123, 124]. In addition, endogenous TGF-β is required for differentiation of $CD14^+$ monocytes into dendritic cells and preferentially increases differentiation of $CD34^+$ hematopoietic stem cells (HSC) into Langerhans cells, the dendritic cell subtype of the skin [125]. Monocytes can also be differentiated into osteoclasts, the reabsorbing cells of the bone which can also be induced by TGF-β [126]. This suggests that endogenous TGF-β plays an important role in differentiation of myeloid cells. However, it seems that full differentiation requires other factors that are present in the surrounding environment, which will change depending on the tissue involved and the degree of inflammation.

TGF-β has a variety of functions in many different cell types, which require the activation of multiple signalling pathways. The main signalling pathway activated by TGF-β is the SMAD (mothers against decapentaplegic homolog) pathway, in particular SMADs 2-4 (Figure 1.7). TGF-β binding to the type II receptor allows recruitment of the type I receptor into the complex [127]. This receptor activation induces phosphorylation and activation of SMAD proteins 2 and/or 3 [128, 129]. Classically SMADs 2 and 3 will form a complex with SMAD4 and migrate to the nucleus and bind to SMAD response elements in the DNA to regulate specific gene expression [129, 130]. However, TGF-β can also signal independently of SMADs via the NFκB, PI3K, ERK1/2 and p38 mitogen activated protein kinase (MAPK) pathways (Figure 1.7) [131-134].

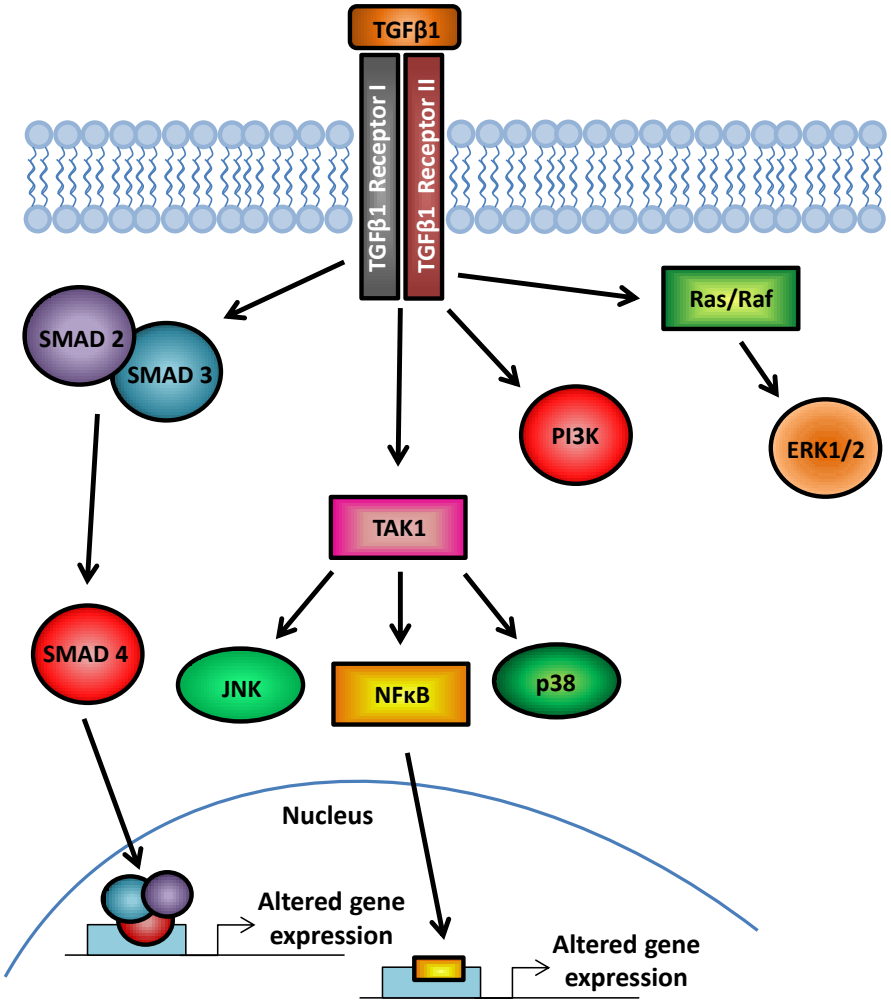

Figure 1.7: TGF-β1 signalling pathway. TGF-β1 activates the TGF-β1 receptor to activate the SMADs 2, 3 and 4. Alternatively, TGF-β1 activates PI3K and Ras/Raf/ERK1/2 signalling pathways and TAK1 resulting in JNK (c-Jun N-terminal kinase 1), NFκB and p38 activation.

Despite these anti-inflammatory mechanisms, in some situations, the inflammation cannot be resolved. This is called chronic inflammation and leads to uncontrolled repair mechanisms resulting in excessive inflammation, fibrosis and scaring [135]. Chronic inflammation is the major cause and symptom of most diseases, including arthritis [136], asthma [74] and chronic obstructive pulmonary disease (COPD) [137]. However, inflammation is now recognised as a critical cause/symptom of many other diseases that were not been previously recognised to have an inflammatory component including cancer [70], cardiovascular disease [138] and diabetes [75]. Although some diseases of the central nervous system (CNS) such as multiple sclerosis have been known to have an inflammatory component for many years now, the role of inflammation and immune system in other CNS diseases such as

Parkinson's disease is becoming increasing acknowledged. As in the periphery, most diseases of the brain have an inflammatory component including AD [73], Parkinson's disease [139] and stroke [140]. Understanding inflammatory mechanisms and how the brain resolves inflammation is key to finding much needed, novel therapies for neurological disorders.

1.2. Inflammation in the Brain

Inflammation also plays an important role in the CNS. The CNS has its own immune cells, called microglia. These cells are closely associated with neurons, and monitor any changes that occur in and around the neuronal environment, with their main function being to protect neurons [141]. Neurons release or display molecules that signal to microglia about the health of the neuron [142]. In their resting state, neurons display molecules on their surface including CD47 [143] and CD22 [144], to inform the microglia of their healthy state and prevent microglial activation. Following neuronal damage, neurons release other signals including glutamate [145], purines [146] and chemokines [147] that are recognised by microglia and initiate microglia activation. In addition, the absence of signals displayed on healthy neurons also causes activation of microglial cells [144].

Microglia are considered the macrophages of the CNS, and play an important role in clearance of pathogens [148], apoptotic cells [149] and cell debris [150]. Microglia are attracted to an area of damage caused by injury or inflammation by signals including chemokines and nucleotides e.g. chemokine (C-X3-C motif) ligand (CX3CL)-1 [151] and adenosine triphosphate (ATP) [152] released by damaged neurons. Microglia sense these stimuli through their chemokine (C-X3-C motif) receptor 1 and purinergic receptors on the cell membrane, which initiate microglial migration towards the gradient of the stimulus to the damaged area [151, 152]. Damaged neurons present molecules such as phosphatidylserine on their surface which are recognised by microglia to induce phagocytosis of the damaged neuron [153]. Phagocytosis of apoptotic cells is very important, as the release of chemokines, nucleotides and cytokines from damaged neurons can increase inflammation in the damaged area [78] and therefore the regulation of microglia activation is critical. Although microglia are important for dead cell clearance, they can also increase inflammation and tissue damage when over-activated [154]. Activation of microglia directly by pathogens or indirectly through neuronal injury can cause the release of

pro-inflammatory mediators including IL-1β, TNF-α and ROS [155, 156]. These mediators act on neurons to induce injury and apoptosis which results in further secretion of inflammatory mediators, activating microglia further [157, 158]. Therefore, in many neurological conditions including MS and neurodegeneration, inhibition of microglial influx and activation has shown beneficial effects [159-161].

Another glial cell type in the brain called astrocytes also secrete cytokines and chemokines in response to brain damage, but to a lesser extent than microglia [162]. Similarly to microglia, inflammatory mediators secreted by astrocytes contribute to inflammation in the brain during inflammation and disease, but they are also important in repair of the brain [162, 163]. Astrocytes prevent over-activation of microglia, suppressing inflammation by increasing protective genes such as heme oxygenase 1 [164].

In addition to microglia and astrocytes, other cell types in the brain can also contribute to inflammation. Endothelial cells, neurons and oligodendrocytes secrete inflammatory mediators, activating the surrounding glia, increasing local inflammation [165]. Endothelial cells secrete cytokines, chemokines and alter expression of adhesion molecules, increasing infiltration of peripheral immune cells into the CNS [166]. Weakening of the blood brain barrier allows leukocytes, particularly neutrophils and monocytes, to migrate to the inflamed tissue, through the chemical gradient, created by the chemokines secreted by microglia and astrocytes in the damaged area [167, 168]. Once at the site of inflammation these cells can contribute to secretion of pro-inflammatory mediators including cytokines and chemokines. Neutrophils also secrete neutrophil elastase and MMPs that cause tissue degradation, making it easier for leukocytes get to the site of inflammation [169].

1.3. Inflammation and Ischaemia

As mentioned previously, ischaemia is a key initiator of the inflammatory response. Ischaemic conditions including stroke and age related macular degeneration (AMD) of the eye are often associated with increased inflammation and a worse clinical outcome [170-172]. Ischaemia increases inflammation by a variety of different mechanisms. The main mechanism of ischaemia-induced inflammation is through induction of ROS including H_2O_2 and $\cdot O_2^-$ through activation of the mitochondrial election transport chain and xanthine and nicotinamide adenine dinucleotide

phosphate (NADPH) oxidases [173-175]. Ischaemia-induced ROS production causes the release of a variety of pro-inflammatory mediators including IL-8 and TNF-α through regulation of activating protein-1 (AP-1) [4], MAPK [5] and NFκB [59] signalling pathways. Ischemia also increases microglial activation and proliferation which can be detrimental in ischaemic conditions such as stroke and AMD further linking these two processes [176, 177].

1.4. Cerebral Ischaemia

Cerebral ischaemia, or stroke as it is more commonly known, is the 4th leading cause of morbidity in the western world and primary cause of major disability worldwide [178]. The risk of stroke is associated with common factors including: diet, family history and comorbidities e.g. diabetes [179], obesity [180] and hypertension [181]. There are two major types of stroke: thrombolytic (~80% of stroke cases) and haemorrhagic (~20% of stroke cases) [182]. A haemorrhagic stroke occurs when a blood vessel in the brain bursts and is often due to high blood pressure. This reduces the blood supply to certain areas of the brain causing neuronal cell death. A thrombolytic/ischaemic stroke occurs when a thrombus, most commonly in the carotid artery, brakes off from the lining of the artery into the blood stream forming an embolus [183]. The embolus is carried via the carotid artery until it blocks the smaller arteries of the brain [183]. The most common site of obstruction is the middle cerebral artery (MCA), this artery supplies blood to the surface of the lateral hemispheres of the frontal, parietal and temporal cortices [184]. A lack of blood flow to these regions results in symptoms associated with the function of the damaged area, and therefore symptoms commonly include: motor disturbances (particularly of the limbs and face), visual disturbances, headaches, dizziness and speech impairment [185].

Following vessel occlusion, the brain regions supplied by the blocked vessel become starved of nutrients essential for neuronal survival, in particular, oxygen and glucose [186]. Neurons supplied entirely by the blocked artery will form the infarct core, in which all the neurons will die, primarily by necrosis (Figure 1.8) [187]. The area surrounding the core is called the penumbra. Blood flow in this area is reduced to around 20% due to a blockage in one of the main arteries that supplies blood to this region [188]. However, this volume of blood is adequate for neuronal survival for a limited time [188]. The survival of the neurons in the penumbra is dependent on a

number of factors, including the duration of cerebral infarction, the amount and diffusion of toxic molecules released from the necrotic core and the effect of reperfusion [188]. Neurons in the infarct core undergo necrosis, which is a less regulated form of cell death in comparison to apoptosis, and this results in release of toxic molecules (particularly glutamate), which diffuse towards the penumbra causing excitotoxic neuronal cell death [187, 189]. Reperfusion is the restoration of blood flow to the brain, and although this is essential for survival, it brings with it toxicity caused by influx of inflammatory cells and pro-inflammatory mediators from the blood [190]. These molecules can lead to further cell death of the vulnerable neurons in the penumbra [190].

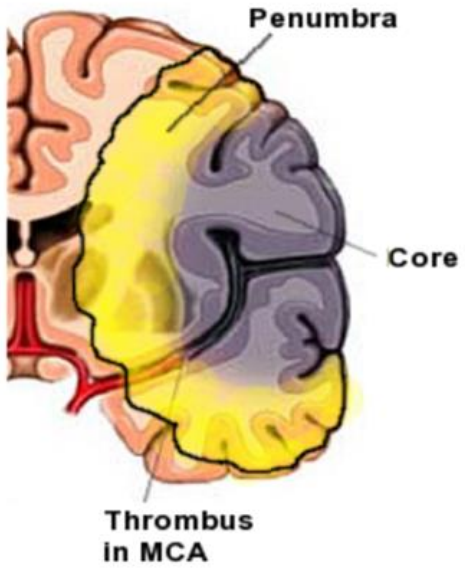

Figure 1.8: Thrombotic occlusion of the MCA. Artery occlusion results in an ischaemic core highlighted in grey with surrounding penumbra in yellow. Figure modified from Mongia et al 2013 [191].

Much of the damage that occurs in the brain after stroke is caused by inflammation. Similarly to peripheral inflammation, recovery of the brain after stroke is also thought to occur in two phases [192]. The first phase begins immediately after vessel occlusion and is characterised by inflammation, ROS production and neuronal cell death [192]. In the second phase, the brain begins to repair the damage caused by the occlusion and Phase I, and this latter phase begins days after vessel occlusion [193]. During this phase, growth factors, anti-inflammatory mediators and anti-oxidant

molecules are released into the damaged area in an attempt to reduce inflammation and increase neuron and vessel repair and regeneration.

1.5. Inflammation in Cerebral Ischaemia

As mentioned previously, inflammatory mediators released during brain inflammation including cytokines and chemokines are also secreted from resident cells of the brain including microglia [194], astrocytes [195], oligodendrocytes [196], endothelial cells [197] and to some extent neurons [198]. Following stroke, nutrient deprivation and neuronal cell death initiates the activation of these cells causing the release of pro-inflammatory molecules in an attempt to initiate the 'clear-up ' or repair the damaged cells [199].

Microglial cells have both neuroprotective and neurotoxic actions in the brain and therefore their role in recovery of stroke is controversial. Intravenous transplantation of the human microglial cell line HMO6, reduced infarct volume and increased neuronal recovery in an animal model of stroke [200]. Microglial cells also increase neural precursor proliferation [201] and regenerate and increase turnover of overactive synapses [202] in stroke, demonstrating their neuroprotective function. On the other hand, activated microglia increase production of ROS, proinflammatory cytokines, chemokines, cytochrome c and caspase 3 resulting in reduced neuronal viability [203, 204]. This suggests that microglia play both beneficial and detrimental roles in stroke, which is likely to depend on the individual and the duration of occlusion and reperfusion.

Peripheral leukocytes infiltrate the brain following stroke due to the weakening of the blood brain barrier that occurs following cerebral ischaemia [205]. Leukocytes in peripheral arteries undergo a series of attachments to the underlying endothelium, by binding to adhesion molecules including integrins, selectins and the immunoglobulins [206]. ICAM-1 is an adhesion molecule increased following stroke [207]. ICAM KO mice survive longer when subjected to middle cerebral artery occlusion (MCAo) compared to wild type animals highlighting contribution of this molecule to cell death in the brain following stroke [208]. The main peripheral inflammatory cell to infiltrate the brain following stroke is the neutrophil [209]. IL-8 is the main chemokine responsible for infiltration of neutrophils following stroke [210]. Neutrophils secrete proteases and pro-inflammatory mediators that increase inflammation and neuronal damage further (Figure 1.9) [211]. T-cells also infiltrate the

brain following stroke and are likely to contribute to the secretion of pro-inflammatory mediators and inflammatory cell infiltration [212]. Leukocyte infiltration into the brain following stroke alters the osmolality of the tissue increasing fluid uptake into the areas of the brain with increased numbers of leukocytes causing brain oedema [205] Oedema increases pressure in the brain, which causes further neuronal cell death, and in extreme cases, can be fatal [213]. In severe cases, external ventricular drainage can be used to reduce oedema to reduce the risk of brain damage [213].

Immune cells secrete pro-inflammatory cytokines including TNF-α [214], IL-6 [215] and IL-1β [216] which are detrimental in the early phases of stroke (Figure 1.9). TNF-α is increased in the brain following stroke and is a major contributor to neuronal cell death [214]. IL-1β is also increased following stoke where it activates immune cells, which in the case of neutrophils, causes the release of MMP-9, which degrades the extracellular matrix and increases brain damage after stroke [217]. Inhibition of MMP-9 [218], inflammatory cytokines [219, 220] or neutrophil influx into the brain [221], reduces infarct volume after stroke, demonstrating the damaging effects of these molecules. However, in the regeneration phase following stroke, MMPs can be beneficial. MMPs are required for breakdown of the extracellular matrix within the infarct, which allows for growth of new neurons and blood vessels and which should be considered when selecting MMPs as therapeutic targets for stoke [222]. IL-1RA is also increased in stroke where it reduces neuronal damage through inhibition of the pro-inflammatory actions of IL-1β [216, 223, 224]. IL-6 on the other hand has demonstrated both beneficial and detrimental effects in stroke. In an animal model of stroke, administration of IL-6 has been shown to reduce cerebral damage [225, 226] conversely, serum IL-6 levels are positively correlated with stroke severity, suggesting that the role of IL-6 in stroke is likely to be both good and bad depending on concentration, time and the nature of the surrounding environment [215, 227].

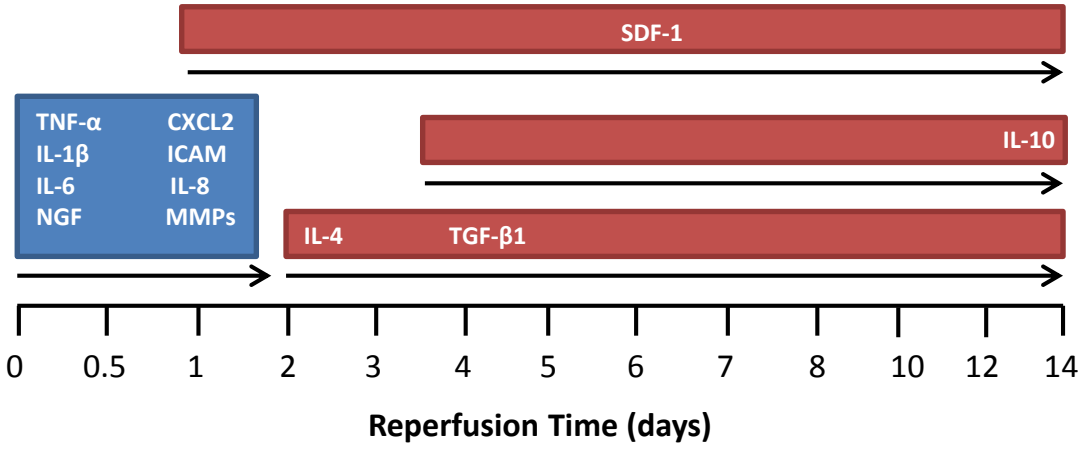

Figure 1.9: Inflammatory protein expression in the brain following stroke. TNF-α, CXCL2, IL-1β, ICAM, IL-6, IL-8, nerve growth factor (NGF) and MMPs are increased within the first 24 h of stroke. Stromal derived factor -1 (SDF-1), IL-10 and TGF-β1 are increased in the days following stroke.

Chemokines are another key group of mediators increased following stroke. Chemokines are the primarily responsible for peripheral leukocyte influx into the brain following stroke [210, 228]. IL-8, CXCL2 and SDF-1 are important chemokines that function to increase leukocyte influx into the brain, which as discussed previously is a major cause of inflammation and brain damage in stroke [168, 228, 229]. However, some chemokines e.g. CX3CL1 have been shown to be beneficial in stroke showing that similarly to other inflammatory mediators, chemokines can have both protective and detrimental effects in stroke [230].

Anti-inflammatory cytokines including TGF-β and IL-10 are also upregulated following stroke (Figure 1.9) [231-233]. IL-10 reduces inflammation via inhibition of IL-12, IL-6, IL-8 and TNF-α activity [234]. In addition, administration of IL-10 in the MCAo mouse model reduces infarct volume, further supporting a protective role for this cytokine in stroke [235]. TGF-β has also demonstrated a protective function after stroke. The anti-inflammatory cytokine and growth factor is upregulated in the brain tissue peaking 7 days after reperfusion in the MCAo mouse model of stroke (Figure 1.9) [236]. As well as having anti-inflammatory properties, such as suppression of immune cell activation and cytokine secretion, TGF-β regulates regeneration of neurons and blood vessels following stroke [237-239].

In summary, immune cell activation causes the release of many pro-inflammatory mediators and increases brain damage following stroke, however, some immune cells including microglia and astrocytes also play a role in repair and regeneration of

the brain following stroke. Much is known about the initial stages of inflammation following stroke, however the mechanisms of repair in the later stages of stroke require further validation. The identification of new biomarkers for this later phase will be a useful tool for the research of inflammation in the brain following stroke.

1.6. TREM-2 and Ischaemia

Due to the need to identify new biomarkers in inflammation following stroke, our lab, in collaboration with Prof. Otto Witte and Dr. Christiane Frahm at the University of Jena, investigated gene expression changes in the brains of mice exposed to MCAo. Mice were subjected to MCAo for 30 min followed by reperfusion for 2 or 7 days (Figure 1.10). Microarray analysis of brain tissue from ipsilateral and contralateral hemispheres resulted in the identification of a potential novel therapeutic target for stroke, a molecule called triggering receptor expressed on myeloid cells -2 (TREM-2). TREM-2 was one of the highest upregulated genes (6-fold) in the repair phase (7 days) following MCAo. TREM-2 belongs to a family of receptors known to play a role in the inflammatory response. TREM-2 itself has demonstrated anti-inflammatory properties [(240)], is regulated by inflammation [(241)] and its expression is altered in various inflammatory conditions [(242-244)]. This suggests that TREM-2 is an important regulator of the inflammatory response.

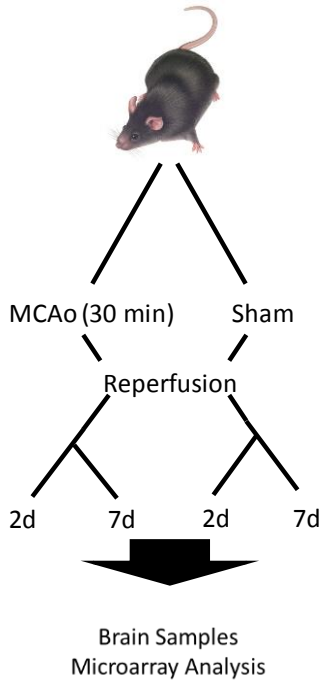

Figure 1.10: MCAo mouse model of stroke. Mice were either sham operated (Sham) or had their MCA occluded for 30 min. Brain samples were collected for microarray analysis 2 or 7 days after reperfusion.

1.7. Introduction to TREM-2 and the TREM Family

1.7.1. The TREM Family

The TREM proteins are transmembrane receptors of the Ig superfamily known for their role in modulation of the inflammatory response [(245)]. They are mainly found in myeloid cell types, although expression in a limited number of other cell types has been documented. The expression of the TREM proteins and their role in inflammation in different cell types varies depending on the TREM protein involved [(245)]. The TREM gene cluster is found on chromosome 6p21.1 and contains TREMs 1-5 and the TREM-like transcript (TLT) proteins (Table 1.1) [(246)]. TREMs 4 and 5 have currently only been identified in the mouse genome and very little is known about these receptors [(246)]. The TLT proteins have a degree of homology to the TREM proteins and have also demonstrated immune regulatory properties amongst other functions (Table 1.1) [(245-248)].

TREM-1 is the most characterised TREM protein and is known for its ability to exacerbate the inflammatory response (Table 1.1). Activation of TREM-1 by the

currently unidentified TREM-1 ligand, initiates a pro-inflammatory response, including secretion of IL-1β, TNF-α and IL-8 [249]. In addition, LPS stimulation of human monocytes (*in vitro* and *in vivo)* increases TREM-1 expression, which is important for pathogen-induced cytokine secretion, demonstrated using TREM-1 KO mice or TREM-1 blocking antibodies, which showed that TREM-1 was in important for pro-inflammatory cytokine secretion and increased mortality following in sepsis [250-252]. TREMs -1 and -2 have been identified in both human and mouse genomes [246, 253]. TREM-3, however, is only expressed in the mouse and consists of a single exon in humans, with no mRNA or protein yet detected [254]. However, the limited research on TREM-3 so far suggests the protein has similar pro-inflammatory properties to its close family member, TREM-1 [248].

Table 1.1: TREM family expression and function.

TREM family member	Species	Expression	Function	Refs
TREM-1	Human, mouse	Neutrophils, monocytes, macrophages, endothelial cells and some epithelial cell types	Pro-inflammatory	(241, 255, 256)
TREM-2	Human, mouse	Microglia, monocyte derived macrophages, hepatic macrophages, dendritic cells, subsets of monocytes, osteoclasts, genitourinary and fallopian tube epithelial cells, endothelial cells, fibroblasts Low levels: some neurons and oligodendrocytes	Anti-inflammatory, phagocytosis, osteoclastogenesis	(240, 241, 243, 244, 257-265)
TREM-3	Mouse	Macrophages, endothelial cells	Pro-inflammatory	(241)
TREM-4	Mouse	Unknown	Unknown	(246)
TREM-5	Mouse	Unknown	Unknown	(246)
TLT-1	Human, mouse	Platelets, megakaryocytes	Osteoclastogenesis, platelet aggregation	(266-269)
TLT-2	Human, mouse	CD8+ T-cells, B-cells, alveolar and peritoneal macrophages, neutrophils	Pro-inflammatory, increases neutrophil activity and recruitment	(247, 270, 271)
TLT-3	Human	Monocytes, B-cell subsets, fibrosarcoma	Unknown	(246)

TLT-4	Human, mouse	Macrophages, dendritic cells	Apoptotic cell recognition, antigen presentation	[272, 273]
TLT-5	Human	Not expressed	Pseudogene	[246]

1.7.2. TREM-2 Expression

TREM-2 is a 230 amino acid glycosylated receptor of the TREM family, and unlike other TREM proteins has anti-inflammatory activity. TREM-2 has been identified on the cell surface of myeloid cells including macrophages, microglia, osteoclasts and dendritic cells (Table 1.1). Evidence suggests that TREM-2 is mainly expressed on amoeboid microglial cells, and that in these cells, TREM-2 is located on both the cell surface and intracellularly [274-276]. High expression of TREM-2 in these amoeboid cells is probably due to the phagocytic and migratory phenotype of these cells, as TREM-2 has been shown to be involved in both of these processes in microglia [81]. TREM-2 expression has also been identified in human fallopian tube and genitourinary epithelial cells [257], mouse liver endothelial cells [241], mouse oligodendrocytes, low expression in neurons [258, 259] and more recently in human fibroblasts [244] (Table 1.1). TREM-2 protein expression is generally low in monocytes [246, 262, 277], but has been shown in some conditions to be expressed at higher levels, for example, on monocytes isolated from the cerebrospinal fluid (CSF) of MS patients [278]. This suggests that basal TREM-2 expression is regulated by changes in the surrounding environment and possibly also by the differentiation state of the cell.

1.7.3. The Role of TREM-2 in Nasu-Hakola Disease

The current knowledge of TREM-2 function has been supported by studies using patients with Nasu-Hakola Disease (NHD) also called polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL). This is a rare, inherited, autosomal recessive disease caused by a deficiency in either the TREM-2 protein or the TREM-2 adaptor protein TYRO protein tyrosine kinase binding protein (DAP12) [279]. Table 1.2 details the types of TREM-2 mutations that have been identified in NHD and the functional effects observed. The first symptoms of NHD most commonly present themselves during adolescence and include: pain and swelling of the ankles and feet and bone fractures [280]. These symptoms occur as a

result of the development of cysts in the bone marrow of long bones caused by a lack of TREM-2 signalling, altering osteoclast formation and development. [260, 279]. The neurological symptoms of NHD include: personality and behavioural changes, in particular, social inhibition, unrestrained behaviour and euphoria, alterations in speech speed developing onto aphasia of speech, and as the disease develops, motor disturbances and memory loss and in some cases epilepsy [280]. These symptoms occur as a result of the following changes in brain physiology: degeneration/atrophy of cortical white matter, demyelination and axon degeneration, enlarged brain ventricles, hypoperfusion of the brain, proliferation of microglia and astrocytes and basal ganglia calcification [279, 280].

Table 1.2: TREM-2 mutations in NHD

TREM-2 DNA Mutation	TREM-2 protein change	Homozygous/ Heterozygous	Functional effects	Refs
C to T mutation at position 97	Q33Stp	Homozygous	Actin filament changes. Increased expression of genes involved in inflammation and immune response and reduced expression of the GABA family and synaptic proteins	[276, 281]
T to G mutation at position 377	V126G	Homozygous	Actin filament changes. Increased expression of genes involved in inflammation and immune response and reduced expression of the GABA family and synaptic proteins	[276, 281]
Splicing mutation: T to C at the second position of intron 3 in the splice-donor consensus site (482)	Truncated proteins	Homozygous	Astrogliosis, demyelination, axonal spheroid formation and basal ganglia calcification.	[282]

Gene expression studies in NHD patients have improved our understanding of TREM-2 function. Analysis of NHD brain cortical tissue showed altered expression of hundreds of genes, many of them involved in inflammation, cellular movement and immune trafficking [282]. Of the genes downregulated, the gamma-aminobutyric acid (GABA) receptor family occurred most frequently [282] which may be why some patients with NHD experience epilepsy, through over-excitation of neuronal circuits. In addition, IL-18, a member of the IL-1 family, was increased by 10-fold in NHD patients [282]. This increase in IL-18 signalling may be in part responsible for the

changes in brain homeostasis observed in NHD [282]. In addition, this study also suggests that TREM-2 is important for preventing over-expression of IL-18 dampening down the inflammatory response in the brain [282].

NHD patients also have reduced expression of genes involved in neurogenesis and synaptogenesis e.g. neuritin 1 and synaptoporin [282], processes that are important in the repair of the brain and spinal cord in disease and following injury [283, 284]. The downregulation of these genes in NHD suggests that the lack of TREM-2 signalling reduces neurogenesis and synaptogenesis, reducing the brains natural repair mechanisms. This is likely to play a role in the neurodegeneration observed in NHD patients and further supports the role of TREM-2 in resolution and repair of the brain. Dendritic cells from patients with NHD also have altered gene expression [281]. The most upregulated genes include: allograft inflammatory factor 1 (AIF1), CCL18, CCL2, CCL23, sialic acid binding Ig-like lectin 1, complement component 1qa, complement C2 and complement component 1qB [281]. All of these genes are involved in inflammation and/or phagocytosis, suggesting that TREM-2 regulates these genes to mediate its anti-inflammatory and phagocytic effects.

In summary, the identification of a defect in TREM-2 in NHD has been a useful tool to better understand the functions of TREM-2 in health and disease.

1.7.4. *TREM-2 Signalling*

1.7.4.1. The TREM-2 Ligand

The current evidence suggests that TREM-2 has multiple ligands, similar to that of the pathogen recognition receptors. A TREM-2 ligand has been identified in neurons, macrophages, dendritic cells and astrocytes [149, 265, 285-287]. These studies measured the TREM-2 ligand using a TREM-2-Fc fusion protein, for this amino acids 19-171 of TREM-2 were cloned with an IgG Fc domain and binding of the fusion protein to the surface of cells was measured by fluorescent antibody binding to the Fc domain [285]. Interestingly, TREM-2 ligand expression is increased on the surface of apoptotic neurons and initiates TREM-2 mediated phagocytosis of apoptotic neurons by microglial cells [149]. The presence of the TREM-2 ligand on TREM-2 expressing cells including macrophages and dendritic cells suggests that these cell types are able to activate TREM-2 independently via ligand secretion or direct interaction with the TREM-2 ligand on the cell membrane.

HSP60 is one protein that is thought to be an agonist for the TREM-2 receptor. HSP60 increased phagocytosis in TREM-2 overexpressing cells, which could be suppressed with downregulation of TREM-2 [286]. However, its low binding affinity for the TREM-2 receptor suggests that it is only effective when microglia are close to the source of HSP60 [286]. TREM-2 ligands have also been reported on the surface of bacteria including *E. coli, S. aureus* and *N. gonorrhoea* but the specific molecules required for TREM-2 activation have only been recognised on *N. gonorrhoea,* identified as lipooligosaccharides [257, 285]. N'Diaye and colleagues also showed that TREM-2 was required for binding and phagocytosis of bacteria in Chinese hamster ovary (CHO) cells, but identified bacterial ligands have not yet been directly linked to TREM-2 phagocytosis [261]. This study further suggests a role for TREM-2 in the clearance of pathogen infection. However, the lack of a TREM-2 ligand makes it more challenging to study its function. The most common way to activate the receptor is through cross-linking with antibodies against TREM-2. It seems that at least for some functions, both F(ab) fragments of the antibody are required for activation, suggesting that more than one receptor is required for TREM-2 activation [262]. In addition, the function of TREM-2 has also been studied using TREM-2 KO animals and has provided great insight into the functions of TREM-2. However, altering TREM-2 expression may not correlate to TREM-2 activity and therefore more research is required to discover and understand TREM-2 ligands, and this will further help understand the diverse functions of TREM-2.

1.7.4.2. TREM-2 Receptor Structure and Signalling

Similarly to other receptors of the Ig superfamily, TREM-2 is a single transmembrane-spanning receptor [246]. TREM-2 has a very small cytoplasmic domain and so requires the adapter protein DAP12 for signal transduction [288]. DAP12 is an adaptor protein for multiple cell surface receptors including signal regulatory peptide beta (SIRP-β), TREM-1 and myeloid DAP12-associating lectin-1 (MDL-1) [262]. DAP12 is a transmembrane protein with a cytoplasmic tail containing an immune tyrosine receptor based activation motif (ITAM), important for association with the TREM-2 receptor (Figure 1.11). The ITAM motif is the site of DAP12 tyrosine phosphorylation following TREM-2 receptor activation [265, 289]. Phosphorylation of tyrosine residues 65 and 76 are important for recruitment,

phosphorylation and activation of Src tyrosine kinases, such as spleen tyrosine kinase (Syk) which bind to the tyrosine residues through their Src homology 2 (SH2) domains (Figure 1.11) [262, 289-291]. TREM-2 has also been shown to associate with DAP10, a similar adaptor molecule with a tyrosine based motif (YINM) [292]. The YINM motif is important for association with the SH2 domain of the PI3K p85 subunit [293]. Phosphorylation of PI3K requires both DAP12 and DAP10 and results in the activation of AKT, Grb2 and ERK1/2 signalling pathways [292]. PI3K activation is also required for Syk phosphorylation, suggesting that PI3K is important for the recruitment and activation of Syk by DAP12 [292].

Studies have also shown ERK1/2 activation downstream of TREM-2 signalling through direct activation of the TREM-2 receptor. Knocking out the TREM-2 receptor reduced ERK1/2 phosphorylation induced by TREM-2 activation using a TREM-2 antibody [260, 291]. Along with protein tyrosine kinases, TREM-2-induced ERK1/2 activation has also been linked to the effects of TREM-2 on dendritic cell maturation and survival, suggesting that TREM-2 mediates its effects on these processes via ERK1/2 [262]

SH2-containing inositol phosphatase (SHIP) has been identified as an inhibitor of these signalling pathways, binding to phosphorylated tyrosines on DAP10 and DAP12 to inhibit binding of other SH2 domain containing proteins including PI3K and Syk [292]. In addition, SHIP-1 KO increases the ability of TREM-2 to potentiate osteoclastogenesis, suggesting that SHIP acts as a negative regulator of TREM-2 signalling [292]. Interestingly, activation of TREM-2 increases SHIP-1 phosphorylation, demonstrating a negative feedback loop to prevent over-activation of the TREM-2 receptor [292].

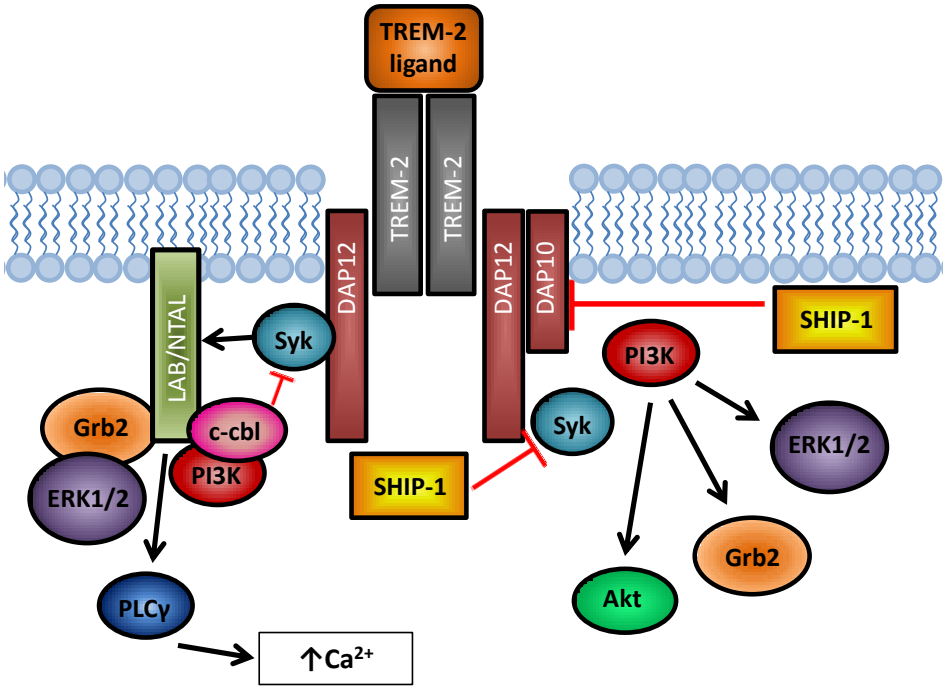

Figure 1.11: TREM-2 signalling cascade. TREM-2 activation allows association with, activation and phosphorylation of DAP12 and or DAP10. This allows PI3K and Syk to bind, which in turn activates other kinases; LAB and ERK1/2, Grb2, PI3K, c-cbl and PLCγ.

The signalling molecule linker for activation of B cells (LAB), also called LAT2 or NTAL, is strongly expressed in macrophages, and is required for TREM-2 activation of ERK MAP kinase in macrophages [(291)]. Following TREM-2 activation, Syk phosphorylates LAB, allowing Grb-2 to associate with the LAB adaptor protein (Figure 1.11). LAB-induced Grb-2 aids recruitment of ERK1/2, c-cbl and PI3K to LAB. LAB activation of c-cbl acts as a negative feedback mechanism to reduce over-activation of TREM-2 by causing ubiquitination and degradation of Syk [(291)]. LAB activation also indirectly activates phospholipase C (PLC)-γ, this leads to an increase in intracellular calcium via diacylglycerol, which may contribute to, or be the initiator of the increased intracellular calcium seen after TREM-2 activation [(294)]. In support of this, PLC has been shown to be recruited to the TREM-2 signalling complex after ligation of the TREM-2 receptor [(292)].

TREM-2 has also been shown to associate with Plexin-A1 (PA-1). The PA-1 receptor is involved in functions including cell migration, angiogenesis, generation of agonist specific T-cells, neuronal out growth, adhesion and spreading [(295)]. Semaphorin 6D (Sema6D) is a ligand for PA-1 and requires TREM-2 expression to initiate PA-1-induced Rac-1 activation [(296)]. However, DAP12, the adaptor molecule 'essential' for TREM-2 signalling seems not to be required for PA-1 activation by

Sema6D, suggesting that in the presence of a co-receptor, TREM-2 can signal without DAP12 [296]. Rac-1 was also identified as an important signalling molecule for TREM-2-induced phagocytosis, in addition to cell division cycle 42 [261]. This suggests that TREM-2 can signal with PA-1 as co-receptor, and therefore may also signal with other receptors. Signalling in this way increases the potential functions of TREM-2.

Peng and colleagues have recently identified the signalling mechanism required for suppression of LPS signalling by DAP12 [297]. LPS induced phosphorylation of docking protein 3 (DOK3) via Src which leads to its translocation to the plasma membrane where it associates with DAP12 and Grb2 [297]. This interaction is required for the ability of DAP12 to supress LPS-induced ERK1/2 activation [297]. This study suggests that the TREM-2 DAP12 signalling pathway suppresses LPS signalling through DOK3 and Grb2 proteins. However, the direct role of TREM-2 in this pathway was not investigated in this study.

Activation of TREM-2 also increases intracellular calcium. Calcium influx induced by TREM-2 cross-linking is dependent on the presence of both F(ab') fragments of the antibody linked together, suggesting that TREM-2 requires two receptors for signal transduction [262]. This increase in intracellular calcium also activates calcineurin, initiating translocation of nuclear factor of activated T-cells c1 (NFATc1) to the nucleus [260, 298]. Similarly to TREM-2, NFATc1 is important in osteoclastogenesis. Recent studies have shown that TREM-2 activation increases NFATc1 expression, and that RANK-induced NFATc1 expression requires upregulation of TREM-2 [260, 298], further suggesting that the interaction between NFATc1, TREM-2 and RANK are important for the regulation of osteoclastogenesis.

In summary, TREM-2 has been shown to associate with other receptors including plexin-A1 and adaptor molecules including DAP12, DAP10 and LAB. It seems that association with different receptors and adaptor molecules alter the downstream signalling cascade activated by TREM-2. This is likely to be critical for understanding the varied functions of TREM-2 in different cell types.

1.7.5. TREM-2 is Important for Differentiation of Myeloid Cells

TREM-2 is increased during myeloid cell differentiation into macrophages, dendritic cells and osteoclasts [240, 299, 300]. TREM-2 is important for proliferation of osteoclasts

precursors and therefore deficiency in TREM-2 results in increased bone resorption due to reduced proliferation and increased differentiation of osteoclasts resulting in bone fragility as observed in NHD [301]. TREM-2 is increased following differentiation of monocytes into dendritic cells [281] and macrophages, particularly M2-type macrophages, which requires IL-4 and M-CSF [240, 302]. The high expression of TREM-2 on these M2-type macrophages has led to the use of TREM-2 as a marker of this cell type [303-305]. In addition, KO of TREM-2 and DAP12 in monocytes does not affect the ability of monocytes to differentiate into dendritic cells *in vitro*, but does prevent correct differentiation into macrophages [281]. Although TREM-2/DAP12-deficient monocytes can differentiate into dendritic cells, there are some differences in cell morphology such as increases in cell surface area and number of processes, highlighting the importance of TREM-2 in myeloid cell maturation [281]. TREM-2 is also increased in differentiation of primary monocytes into dendritic cells with IL-4 and GM-CSF [299]. In addition, TREM-2 ligation in dendritic cells induces dendritic cell maturation measured through upregulation of molecules required for T-cell activation including major histocompatibility complex (MHC) class II, CD40 and CD86 further supporting a functional role for TREM-2 in dendritic cell maturation and differentiation [262]. Taken together, this shows that TREM-2 is in important for differentiation and maturation of dendritic cells and macrophages, and may also be a useful marker for myeloid differentiation [262].

Macrophages deficient in DAP12 have reduced expression of E-cadherin and dendritic cell specific transmembrane protein (DC-STAMP) [306]. These molecules are dendritic cell markers, further supporting a role for the TREM-2/DAP12 complex in dendritic cell maturation. However, these proteins are also important for cell fusion and E-cadherin also is plays a role in β-catenin homeostasis, which is important in the regulation of the cell cycle [307]. In addition, active DAP12 induces calcium induced calmodulin activation, which is required for phosphorylation and activation of proline rich tyrosine kinase 2 (Pyk2), a kinase important for regulation of beta-catenin function by E-cadherin, further linking DAP12 with this signalling pathway [308]. TREM-2 may be the receptor that binds to DAP12 for activation of this signalling pathway, particularly since TREM-2 activation initiates calcium influx which could activate this pathway. This suggests that the effect of TREM-2 on dendritic cell maturation may be mediated by this calcium-calmodulin Pyk2 signalling pathway.

The upregulation of TREM-2 during monocyte differentiation into macrophages may be in part though H3K4me3 histone modification of TREM-2 during differentiation

[309]. TREM-2 is also regulated by M-CSF, a key mediator in myeloid differentiation that increases TREM-2 expression in myeloid cells [240]. One transcription factor that both M-CSF and GM-CSF activate is PU.1 (SPI1; spleen focus forming virus (SFFV) proviral integration oncogene). The effect of M-CSF and GM-CSF on myeloid differentiation is inhibited in PU.1 deficient mice [310, 311]. Interestingly, PU.1 has two predicated binding sites on the TREM-2 promoter, implicating PU.1 as a regulator of TREM-2 expression in differentiation (Figure 1.12) [298].

Predicted binding sites for microphthalmia-associated transcription factor (MITF) have also been identified on the TREM-2 promoter (Figure 1.12) [298]. MITF is an important factor in myeloid cell differentiation and has been particularly associated with osteoclast function, differentiation and proliferation [312]. The identification of a predicted MITF binding site on the TREM-2 promoter suggests that this gene regulates TREM-2 expression and this may be how MITF regulates osteoclast function.

GATA binding protein 1 (GATA1), runt-related transcription factor 1 (RUNX1/AML-1), C/EBPα and myogenic differentiation 1 (MyoD) also have predicted binding sites on the TREM-2 promoter (Figure 1.12) [298]. These molecules are transcription factors that regulate the cell cycle and differentiation [298]. This further supports the important role of TREM-2 in differentiation and cell cycle regulation.

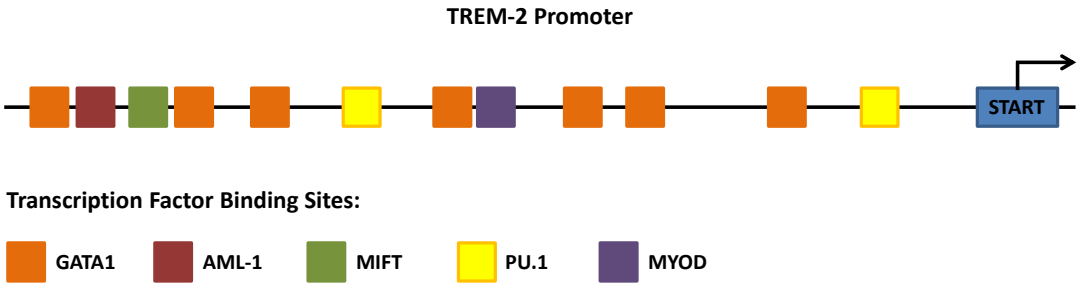

Figure 1.12: Binding sites on the TREM-2 promoter.

In summary, TREM-2 is upregulated during myeloid differentiation. A number of transcription factors may be involved in upregulation of TREM-2 in differentiation including: PU.1, MITF, GATA1, RUNX1, C/EBPα and MyoD all of which have binding sites on the TREM-2 promoter. KO studies in mice have shown the importance of TREM-2 in differentiation of monocytes into osteoclasts,

macrophages and dendritic cells but further research is required to determine the exact role of TREM-2 in these functions.

1.7.6. The Effect of TREM-2 on Phagocytosis

Phagocytosis is the process of engulfment of apoptotic cells, pathogens or unwanted extracellular debris into a cell. Cells that have the ability to phagocytose are called phagocytes and include immune cells including: microglia, macrophages and dendritic cells [313-318]. Phagocytosis of apoptotic cells has been linked to inflammation, injury and neurodegeneration [319, 320]. It has been shown that when this process is diminished, the presence of apoptotic cells and their components exacerbate inflammation and therefore the clearance of these cells is very important for tissue repair and the resolution of inflammation [78]. Apoptotic cells release nucleotides including adenosine triphosphate (ATP) [321] and uridine diphosphate (UDP) [322] and the chemokine CX3CL1 [160] which are sensed by phagocytes to initiate migration towards the dying cells. Identification of apoptotic cells by phagocytes occurs by the appearance of signals on the apoptotic cell membrane including oxidised low density lipoproteins (oxLDL) [323], phosphatidylserine (PS) [324] and collectins [325]. These molecules are identified by the phagocyte and allow the cell to be targeted for phagocytosis (Figure 1.13) [323-326]. The most widely acknowledged marker of cell apoptosis or necrosis is PS. PS binds to cell surface receptors and integrins on phagocytes, initiating an intracellular signalling cascade resulting in the formation of the phagosome resulting in phagocytosis (Figure 1.13) [327-329]. In the CNS, microglia secrete milk fat globule-EGF factor 8 protein (MFG-E8) which aids binding of PS to its receptor on the microglial cell surface to encourage phagocytosis [330]. Although phagocytosis is generally beneficial, during inflammation, phagocytosis can become dysregulated, in these cases, microglia have been shown to phagocytose viable neurons increasing neuronal cell death, highlighting the importance of regulation of this process [331].

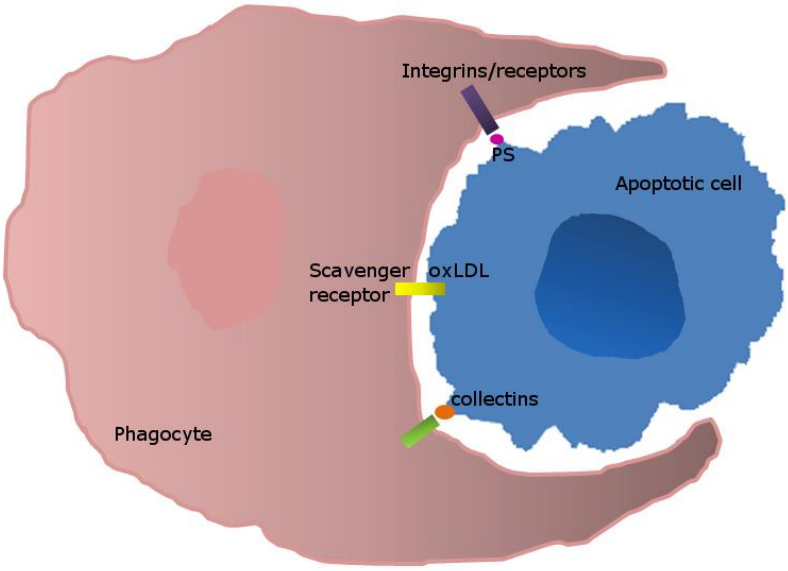

Figure 1.13: Phagocytosis of an apoptotic cell. The apoptotic cell is recognised by the phagocyte due to the appearance of molecules on its surface including PS, oxLDL and collectins signalling to the phagocyte that the cell requires clearance.

There are several lines of evidence showing the importance of TREM-2 in phagocytosis. Expression of the TREM-2 receptor in CHO and mouse microglial cells is required for phagocytosis of *E.coli* [261] and apoptotic neurons, respectively [81]. Phagocytosis of *E.coli* was reduced by 30% in TREM-2 deficient CHO cells [261]. In TREM-2 deficient microglia, phagocytosis of apoptotic neurons was reduced by 58%, suggesting that TREM-2 is involved in phagocytosis, but is not the only mechanism in this system [81, 261]. Activation of TREM-2 in microglial cells also causes f-actin polarisation, suggesting that TREM-2 induces cytoskeletal rearrangement, a requirement of phagocytosis [81]. TREM-2 increases phagocytosis of *E.coli* in CHO cells by activation of the Ras homolog family member (Rho) family GTPase's: Rac-1 and cell division cycle 42 (cdc-42) and in part by PI3K [261]. This suggests that activation of these signalling molecules are important for TREM-2 mediated phagocytosis. Finally, phagocytosis is inhibited in beclin 1 KO mice, which have reduced recycling of TREM-2 due to downregulation of retromer receptor recycling machinery [332], suggesting that beclin 1 regulates phagocytosis in part though TREM-2 receptor recycling [333].

Altogether, the information summarised here shows that TREM-2 is important in phagocytosis of bacteria and apoptotic cells. The presence of apoptotic cells, bacteria and cell debris increases inflammation in tissues which highlights the importance of TREM-2 in the resolution of inflammation.

1.7.7. The Role of TREM-2 in the Inflammatory Response

1.7.7.1. Inflammatory Changes Induced by TREM-2 Signalling

TREM-2 has been recognised for its potential to modulate inflammation and the immune response (Table 1.3). In macrophages, TREM-2 activation causes nitric oxide (NO) release [288]. However, in a model of wound recovery and in microglia neuronal co-culture, TREM-2 KO mice have increased expression of inducible nitric oxide synthase (iNOS) suggesting that NO is in fact reduced following TREM-2 activation [81, 334]. This suggests that the regulation of NO by TREM-2 is varied, which is likely to depend on the mediators in the surrounding environment. TREM-2 has also been shown to regulate ROS, another mediator involved in microbial killing (Table 1.3). DAP12 KO and TREM-2 knockdown (KD) macrophages both have reduced ROS production induced by the bacterium *Salmonella enterica serovar Typhimurium* which in DAP12 KO macrophages is associated with increased bacterial burden and inflammation [335]. This suggests that the increase in ROS-induced by TREM-2/DAP12 activation is important for bacterial killing and that reducing the ability of TREM-2 to kill bacteria increases inflammation.

Table 1.3: Effect of TREM-2 expression and activation on inflammation. siRNA, small interfering ribonucleic acid; NK, natural killer; CCR, chemokine (C-C motif) receptor ; KD, knockdown; shRNA, small hairpin RNA; NLR, Nod-like receptor; MR, mannose receptor.

Tissue or cell type	Conditions	TREM-2 activity	Effect	Refs
Mouse colon tissue	Colonic mucosal wound repair	KO	Increased iNOS and reduced arg-1 and MR	[334]
Primary murine microglia	Cultured in the presence of apoptotic neurons	Overexpressed	Increased CCR7 and reduced iNOS, IL-1β and TNF-α	[81]
MT2 macrophage cell line	Cell line transfected with flag tagged TREM-2a	Activated	NO release	[288]

RAW 264.7 macrophages and primary murine macrophages	*Salmonella enterica serovar Typhimurium* induced ROS	TREM-2 shRNA (RAW 264.7) DAP12 KO (primary macrophages)	TREM-2 KD and DAP12 KO; reduced ROS. DAP12 KO mice: reduced bacterial killing and increased inflammation (not studied in TREM-2 KD)	(335)
Dendritic cells	IL-4 stimulation	TREM-Fc to block TREM-2 activation	Reduced bacterial killing and IL-4-induced NK cell activation	(336)
Dendritic cells	TLR and NLR activation	TREM-2 KO mice	Reduced IL-12, TNF-α, IL-1β and IL-6 secretion and reduced dendritic cell induced T-cell proliferation, priming and bacterial killing	(337)
Dendritic cells	TLR activation	TREM-2 KO	Increased TLR-induced IL-12, TNF-α, IL-10 and IL-6	(338)
Mouse macrophages	TLR and Fc activation	DAP12 KO or TREM-2/DAP12 chimera KD and KO of TREM-2	DAP12 KO: increased TLR and FcγR-induced TNF-α secretion. Reduced TLR induced TNF-α secretion with TREM-2/DAP12 chimera. TREM-2 KO increased TLR receptor-induced TNF-α and IL-6 secretion	(240, 265)
Alveolar macrophages	LPS stimulation	TREM-2 shRNA KD	TREM-2 KD increases LPS-induced TLR4 expression and IL-10 and TNF-α secretion	(339)
Microglial cells	TREM-2 high or low microglia added to T-cell culture	shRNA KD of TREM-2	TREM-2 high microglia: increased T-cell proliferation, TNF-α and CCL2 production	(340)
Dendritic cells	TREM-2 ligation with $F(ab')_2$	TREM-2 activation	Increases dendritic cell survival and maturation and MHC class, CD40, CD86 and CCR7 protein expression.	(262)
Dendritic cells	LPS stimulation	TREM-2 KO	Reduced LPS-induced CD86 and CXCL10 expression	(258)
Macrophage	IL-4 stimulation	TREM-2 siRNA KD DAP12 KO macrophages	TREM-2 KD: reduced IL-4-induced macrophage fusion. DAP12 KO: reduced IL-4-induced integrin β3 and cadherin-1 expression.	(306)

Another change observed in TREM-2 KO mice is reduced expression of arginase 1 (arg1) (Table 1.3) [334]. Arg-1 reduces pro-inflammatory cytokine secretion and differentiation into the M2-type macrophage [341, 342]. TREM-2 is also important for M2 type macrophage activation and is even used as a marker of M2 activation in

many studies, showing that it is highly expressed in these cells [334]. This suggests that TREM-2 positively regulates arg-1 expression and that arg-1 may be one of the signalling molecules important for the induction of M2 macrophage activation and anti-inflammatory properties of TREM-2.

TREM-2 has demonstrated anti-inflammatory activity through inhibition of TLR-induced cytokines. TREM-2 activation and TREM-2 KO studies have shown that TREM-2 supresses TLR-induced TNF-α, IL-10, IL-12 and IL-6 secretion [240, 265, 338]. A similar effect was observed with overexpression of TREM-2 in microglia during phagocytosis of apoptotic neurons, resulting in reduced IL-1β, TNF-α and iNOS, providing further evidence for the role of TREM-2 in the resolution of inflammation [81]. The ability of the TREM-2/DAP12 signalling pathway to reduce pro-inflammatory cytokine secretion further demonstrates the anti-inflammatory effects of TREM-2. However, in contrast to these anti-inflammatory effects of TREM-2, Correale and colleagues showed that TREM-2 KO mice had reduced IL-12, TNF-α, IL-1β and IL-6 secretion following TLR and NLR activation in dendritic cells [337]. This suggests that in that system, TREM-2 has a pro-inflammatory effect. However, this study also showed reduced bacterial killing by dendritic cells, supporting previous reports showing the importance of TREM-2 in bacterial killing (Table 1.3) [337]. This contrasting report of the inflammatory effects of TREM-2 suggests that although TREM-2 is anti-inflammatory in many circumstances, in some situations it may be pro-inflammatory.

TREM-2 is also important for dendritic cell function, including IL-4-induced natural killer cell activation and bacterial killing (Table 1.3) [336]. TREM-2 prolongs dendritic cell survival and induces dendritic cell maturation, increasing expression of MHC class proteins, CD40, CD86 and CCR7 [262, 336]. In addition, TREM-2 activation in myeloid cells increases the anti-apoptotic protein, myeloid leukaemia cell differentiation protein (MCL-1), which may be how TREM-2 mediates its pro-survival effects on dendritic cells [292]. The effect of TREM-2 activation on CCR7 expression has also been demonstrated in microglial cells and is required for microglial migration towards the CCR7 ligands, CCL21 and CCL19 revealing a role of TREM-2 in microglial migration [81, 262]. DAP12 has also been shown to be important for migration, in CCL2-induced macrophage recruitment to the lungs following infection, further supporting a role for TREM-2/DAP12 signalling in migration [242]. TREM-2 KO studies have also shown its ability to regulate other chemokines including CD86 and CXCL10 suggesting that TREM-2 is important for the increase in these chemokines after LPS stimulation (Table 1.3) [281]. In addition, CXCL10 increases phagocytosis

[343] and both CD86 and CXCL10 regulate T-cell activity, suggesting that TREM-2 may mediate its effects on T-cell activity, phagocytosis and migration in part by upregulation of CXCL10 and CD86 [344, 345].

Activation of TREM-2 also increases microglial MHC class II protein expression (Table 1.3) [81, 340]. The primary role of MHC class II molecules is antigen presentation. Expression of MHC class II molecules in macrophages allows the macrophage to identify and present the antigen to T-cells to initiate an appropriate antigen response, suggesting TREM-2 may promote or prime the cell for antigen presentation [346]. The role of TREM-2 in T-cell responses is further supported by the ability of the TREM-2 co-receptor PA-1 to regulate antigenic T-cell responses and the reduced T-cell priming in TREM-2 KO mice following ovalbumin sensitisation (Table 1.3) [296, 337]. MR is also involved in antigen processing and is reduced in TREM-2 KO mice (Table 1.3) [334]. MR is important in IL-4 mediated macrophage fusion and antigen capturing [347, 348] two processes that TREM-2 and DAP12 are also important for [306, 349]. In addition, knocking out TREM-2 reduces IL-4-induced integrin β3 and cadherin-1 expression, two membrane proteins known to regulate macrophage fusion [306], suggesting that MR and TREM-2 may be part of a signalling pathway required for cadherin-1 and integrin β3 expression, controlling IL-4 mediated macrophage fusion.

In summary, TREM-2 is important for dampening down pro-inflammatory cytokine secretion, increasing migration and macrophage fusion and regulating antigenic responses. TREM-2 also regulates mediators important for microbial killing including NO and ROS which may help to promote TREM-2-induced phagocytosis. The regulation of these mediators by TREM-2 highlights its important role as a mediator of inflammation and the immune response.

1.7.7.2. Soluble TREM-2

As with other transmembrane receptors, TREM-2 also exists in a soluble form (sTREM-2) [278]. sTREM-2 may originate from a mRNA splice variant that lacks a transmembrane domain and/or though proteolytic cleavage of the TREM-2 protein at the plasma membrane [350]. The sTREM-2 receptor has been identified in CSF of healthy humans and is increased in the CSF of MS patients [278]. Schmid and co-workers showed that the ratio of the murine TREM-2 mRNA: soluble TREM-2

mRNA was 9:1 in unstimulated microglia and macrophages. Following LPS/IFN-γ stimulation the concentrations of both sTREM-2 and membrane bound TREM-2 were reduced, but in macrophages with a ratio of 6:1 and in microglia with a ratio of 14:1 suggesting that double the amount of sTREM-2 is produced in activated macrophages compared to microglia [350], suggesting that sTREM-2 is regulated differently in these cell types. Other soluble receptors such as sIL-1RII, bind to freely available IL-1, reducing IL-1 signalling through its active receptor (IL-1R1) [351]. Although it has not yet been proven, it is likely that sTREM-2 also acts in this way, and therefore may inhibit the beneficial functions that TREM-2 has demonstrated in MS and other diseases.

1.7.7.3. The Role of TREM-2 in Inflammatory Disease

TREM-2 expression is altered in a number of inflammatory conditions including COPD [242], MS [243] and AD [352] (Table 1.4). In AD, this was observed in the APP23 animal model with increased TREM-2 expression in microglia surrounding amyloid beta plaques [352]. In addition, Hu and colleagues have shown that AD patients have increased TREM-2 expression in peripheral blood mononuclear cells (PBMCs) further suggesting that TREM-2 is increased in AD [353]. However, TREM-2 has also been shown to be reduced in the hippocampus of AD patients, which in this study was regulated by mi-RNA-34a [354]. This suggests that either different regions of the brain have varied TREM-2 expression or that the mouse model of AD does not mimic the human disease. In addition, several single nucleotide polymorphisms (SNPs) have now been identified in the human TREM-2 gene that are associated with an increased risk of AD (Table 1.4) [355]. The variant R47H has the most significant association with development of AD and patients with this variant of TREM-2 have increased levels of tau and phosphorylated tau in their CSF [356-358]. In addition, a proteolytic cleavage site has been identified on the extracellular domain of TREM-2 for the AD associated protease γ-secretase [359]. TREM-2 cleavage by γ-secretase is important to sustain TREM-2 activity, shown by a reduction in TREM-2 activity with γ-secretase inhibition, despite increasing TREM-2 surface expression [359]. This study also suggests that the regulation of TREM-2 expression may be more complex than surface expression, and that other factors such as receptor cleavage may be required for TREM-2 activity. It is likely that the function of TREM-2 in AD is through phagocytosis of degenerating neurons and amyloid beta and its anti-inflammatory effects, though this has not yet been studied.

TREM-2 expression is also increased in stroke and MS with opposing effects. In stroke, TREM-2 KO mice have reduced inflammation suggesting a pro-inflammatory effect of TREM-2, but blockade of TREM-2 in MS exacerbates the disease phenotype and increases inflammation suggesting an anti-inflammatory role for TREM-2 [243, 360]. It seems that the role of TREM-2 in neuroinflammation is more complex than originally thought, and that TREM-2 may be pro-inflammatory in stroke and anti-inflammatory in MS. However, despite the reduced inflammatory response in the TREM-2 KO in the MCAo model of stroke, there was no difference in lesion size between wild type and TREM-2 KO mice. Other clinical scores such as motor ability were not measured, therefore the effect of TREM-2 on stroke outcome remains unknown [360].

Table 1.4: Expression and effect of TREM-2 in inflammatory diseases. Positive (+ve) or negative (-ve) effect on inflammation and/or disease symptoms. FTD, Frontal Temporal dementia; RA, rheumatoid arthritis.

Disease	TREM-2	+ve or -ve effect of TREM-2 in disease	Comments	Refs
Alzheimer's Disease	↑ ↓	+ve	APP23 mouse model: 14.1 fold increase on microglia surrounding plaques Cleaved by γ-secretase Reduced in hippocampus of AD patients R47H TREM-2 mutation associated with AD and increased tau in CSF Increased expression on PBMCs in AD	(352-354, 359)
Fronto-temporal Dementia	↑	+ve	R47H TREM-2 mutation associated with FTD	(357, 358)
Parkinson's disease	-	+ve	R47H TREM-2 mutation associated with FTD and Parkinson's disease	(361)
Prion Protein Disease	↑	N/A	ME7 mouse model	(362)
Multiple Sclerosis	↑	+ve	In microglia and macrophages surrounding lesions.	(243)
Peripheral Nerve Injury	↑	N/A	Caused by increased M2 macrophage activation	(303)
Stroke	↑	-ve	Increased 7 and 28 days after MCAo. TREM-2 KO mice: reduced microglial activation and pro-inflammatory cytokines Increased TREM-2 positive macrophages in rat MCAo	(360, 363)

Colonic Wound Repair	↑	+ve	Increased 2 days following injury	(334)
Diabetes	↑	N/A	Db/db mouse: increased H3K4 dimethylation on TREM-2 promoter	(364)
COPD	↑	N/A	Associated with macrophages	(242)
Pulmonary Sarcoidosis	↑	N/A	On bronchial alveolar lavage myeloid cells	(365)
Infection	↑	N/A	*Taenia crassiceps* infection	*(366)*
Rheumatoid Arthritis	↑	N/A	Throughout RA joint	(244)
IBD and Colitis	↑	-ve	TREM-2 KO: reduced disease symptoms, pro-inflammatory cytokines and bacterial killing	(337)
Allergy	↑	N/A	Ovalbumin and house dust mite mouse models of allergy	(240, 367)
Pseudomonas aeruginosa corneal infection	↑	+ve	TREM-2 KD: increased bacterial burden and corneal inflammation. PI3K/AKT pathway required for protective effect of TREM-2	(368)
Experimental autoimmune uveitis	↑	N/A	In eye following experimental autoimmune uveitis	(369)
Polymicrobial sepsis	↑	+ve	In peritoneal fluid, lung, spleen and liver of sepsis patients. TREM-2 blockade: increased bacterial burden and reduced survival. TREM-2 beneficial in model of sepsis	(370)

TREM-2 is also increased in smokers and in patients with inflammatory conditions including COPD [(242)], rheumatoid arthritis [(244)], acute peripheral nerve injury [(303)], *Taenia crassiceps* larvae infection [(366)], diabetes [(364)], inflammatory bowel disease [(337)] and wound healing [(334)] (Table 1.4). Similarly to that seen in the stroke, inflammatory bowel disease patients and animal model of colitis also show a pro-inflammatory effect of TREM-2 (Table 1.4) [(337)]. TREM-2 KO mice had reduced IL-1β, TNF-α, MMP-3 and MMP-9 secretion after dextran sodium sulphate and 2,4,6-trinitrobenzene sulfonic acid-induced colitis [(337)]. In contrast, an animal model of colonic mucosal wound repair showed that TREM-2 is required for efficient wound healing [(334)]. TREM-2 KO in this model of wound repair resulted in dysregulation of the stromal-epithelium interface, which is often associated with inflammatory bowel conditions e.g. Crohn's disease [(371)]. The pro-inflammatory cytokines IFN-γ and

TNF-α were also increased in the wound bed of TREM-2 KO mice [334]. Interestingly antibodies for IFN-γ and TNF-α showed that the ability for TREM-2 to suppress these cytokines was important for the protective effects of TREM-2 in this model, demonstrating that in this model, TREM-2 is important for dampening down pro-inflammatory cytokine secretion in response to injury. The differences between these two models of gastrointestinal inflammation highlight the importance and complexity of TREM-2 in gastrointestinal homeostasis.

TREM-2 is also increased in the peritoneal fluid, lungs, liver and spleen of patients with polymicrobial sepsis [370]. Chen and colleagues also showed that overexpression of TREM-2 in bone marrow derived macrophages increased IL-10 expression and bacterial phagocytosis, and that TREM-2 regulated phagocytosis was dependent on AKT [370]. TREM-2 was also increased in a model of polymicrobial sepsis [370]. In this model, TREM-2 overexpressing bone marrow derived macrophages reduced bacterial burden and increased survival in this model of sepsis, suggesting that TREM-2 is important for bacterial clearance in sepsis [370].

In summary, the expression of TREM-2 is upregulated in many inflammatory diseases. The conflicting evidence between a protective or detrimental role for TREM-2 in these conditions demonstrates the importance of TREM-2 in disease. The disease modifying actions of TREM-2 are likely to be though its ability to modulate the immune response and its role in phagocytosis. However, due to the wide range of conditions TREM-2 has been implicated in, it is likely to have other functions that are yet to be discovered.

1.7.7.4. Regulation of TREM-2 by Inflammatory Mediators

Little is known about the regulation of TREM-2, aside from the ability of particular inflammatory mediators to alter its expression. As shown in Table 1.5, many pro-inflammatory mediators are known to reduce TREM-2 expression including LPS [240, 243], IL-1β, TNF-α [241] and IFN-γ [372]. Vitamin D has also been shown to reduce TREM-2 gene expression in human myometrial smooth muscle cells [373]. In contrast, vasoactive intestinal peptide (VIP) has been shown to increase TREM-2 expression by reversing LPS-induced TREM-2 suppression [374]. VIP inhibits the binding of NFκB to DNA thereby inhibiting the signalling cascade activated following TLR4 activation by LPS [374]. This suggests that the effect of LPS on TREM-2 expression may be dependent on the actions of NFκB. TREM-2 is also increased in

murine microglia cells by the chemokine CX3CL1 secreted from mesenchymal stem cells [375]. Interestingly, the hormones 17β-estradiol and progesterone that have demonstrated neuroprotective properties have also been shown to increase TREM-2 under hypoxic conditions [376].

Table 1.5: Known mediators of TREM-2 expression.

Mediator	Change in TREM-2	Cell type	Refs
IL-27	↓ expression	Human osteoclast precursors	[377]
IL-10	↓ expression	Human $CD14^+$ monocytes	[260]
IL-1β	↓ expression	Murine hepatic endothelial cell and macrophages	[241]
TNF-α	↓ expression	Murine hepatic endothelial cells and macrophages	[241]
IFN-γ	↓ expression	RAW264.7 murine macrophages and human osteoclast precursors	[240, 372]
LPS	↓ expression	RAW264.7 murine macrophages	[240, 263]
Pam_3Cys	↓ expression	Human $CD14^+$ monocytes	[372]
VIP	↑ expression	RAW264.7 murine macrophages	[374]
IL-4	↑ expression	Murine peritoneal macrophages	[240]
M-CSF	↑ expression	Murine peritoneal macrophages	[240]
CX3CL1	↑ expression	N9 murine microglial cells	[375]
Ionomycin	↑ expression	Human CHME-5 microglia and T98G glioblastoma cells	[259]
sTLT-1	↓ activation	Murine bone marrow-derived macrophages	[266]
Aluminium	↓ expression	CB-84 murine microglial cells	[378]
Vitamin D	↓ expression	human myometrial smooth muscle	[373]
17β-estradiol	↑ expression	Murine BV2 microglial cells	[376]
Progesterone	↑ expression	Murine BV2 microglial cells	[376]
Phosphatidylcholine liposome	↑ expression	Murine tumour bearing skin	[379]

As previously discussed, the anti-inflammatory cytokine IL-4 and M-CSF increase TREM-2 expression in monocytes (Table 1.5) [240]. M-CSF is commonly used to differentiate myeloid precursors into macrophages, and evidence suggests that these macrophages have more of an M2 like phenotype than an M1 phenotype [380]. Interestingly, M-CSF alone increased TREM-2 expression in bone marrow derived

macrophages [240]. This may be due to the change in macrophage phenotype towards the M2-type fate particularly since TREM-2 has been used in the literature as a marker of M2 activation [275, 303-305].

The calcium ionophore, ionomycin also increases TREM-2 expression. This was observed on the cell surface of human glioblastoma and microglial cell lines [259]. Ionomycin increases intracellular calcium concentration in the cell through mobilisation of intracellular calcium stores [259]. Interestingly, the increase in TREM-2 expression is calcium dependent, suggesting that calcium influx activates a signalling cascade that regulates TREM-2 surface expression [259]. Signalling molecules activated by intracellular calcium include calmodulin, protein kinase C and calcineurin and therefore may also be involved in TREM-2 regulation.

Aluminium stimulation of microglia also reduces TREM-2 expression [378]. This is particularly interesting as aluminium has been linked to AD risk, further suggesting that TREM-2 function reduces the risk of dementia [381]. In addition, the TREM family member TLT-1 has also been shown to reduce activation of TREM-2 [266]. TLT-1 has a negative effect on osteoclastogenesis and bone reabsorption by inhibiting of AKT phosphorylation after TREM-2 activation by recruiting SHIP-1 to the TREM-2 complex [266]. This may be one of the mechanisms by which TLT-1 negatively regulates osteoclastogenesis.

In summary, TREM-2 is regulated by inflammatory mediators. However, most inflammatory mediators reduce TREM-2 expression and more research is required to understand what mediators cause an increase in TREM-2 expression in disease.

1.7.8. Signalling Pathways Involved in Regulation of TREM-2 Expression

The TREM-2 receptor is thought to be regulated, in part through recycling at the plasma membrane. The TREM-2 receptor is stored in exocytic vesicles that fuse with the plasma membrane after stimulation with mediators known to increase TREM-2 surface expression e.g. ionomycin [264]. The presence of this regulatory activity suggests that the function of TREM-2 is very important and is brought to the membrane quickly following stimulation.

Although TREM-2 is increased in many inflammatory conditions and there are several inflammatory mediators known to regulate its expression, very little is known

about the mechanisms involved in TREM-2 regulation by inflammatory mediators. Inhibition of PI3K has been shown to reduce basal levels of TREM-2, but had no effect on the ability of IL-1β and TNF-α to reduce TREM-2 expression [(241)]. In addition, Alexandrov and colleagues showed that suppression of TREM-2 with aluminium required NFκB and miRNA-34a [(378)]. A similar observation was also demonstrated by Zhao and colleagues showing that in microglial cells, miRNA-34a supressed TREM-2 gene expression [(354)]. However, aside from these signalling molecules, the mechanisms of regulation of TREM-2 remain unknown.

In conclusion, the immunomodulatory and phagocytosis inducing properties of TREM-2 make it a potential candidate as a therapeutic agent for inflammatory conditions. However, although TREM-2 is known to be increased in many inflammatory conditions, most inflammatory mediators have been shown to reduce TREM-2 expression and few are known to increase it. Understanding what induces TREM-2 expression in disease is vital to understand the many functions of TREM-2 in these conditions. Analysing the mechanisms of TREM-2 regulation will also be useful when considering TREM-2 as therapeutic target for inflammatory conditions.

1.8. Aims

The main aims of this study were to:

1. Characterise the expression of TREM-2 in myeloid and non-myeloid cell types.
2. Investigate the effects of pro- and anti-inflammatory mediators on TREM-2 expression in myeloid cells.
3. Examine the mechanisms involved in activation of TREM-2 expression by inflammatory mediators and the functional effects of induced TREM-2 expression.
4. Establish an *in vitro* model of ischaemia to investigate the mechanism of TREM-2 induction following stroke.

2. Materials and Methods

2.1. Reagents

2.1.1. General Reagents

Dimethyl sulfoxide (DMSO), bovine serum albumin (BSA) was purchased from Sigma (Dorset, UK), sterile phosphate buffered saline (PBS) from PAA (Somerset, UK) and paraformaldehyde (PFA) from Fisher (Loughborough, UK). Fibronectin and Vitrogen were purchased from BD Biosciences (Oxford, UK) and deionised H_2O (dH_2O) was collected from the ultrapure water system NANOpure Diamond D11911 (pore size: 0.2 µm) purchased from Fisher.

2.1.2. Stimulators

LPS from *E. coli* O111: B4, peptidoglycan (PGN) and (*S*)-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser(*S*)-Lys_4-OH, trihydrochloride (Pam3Cys) were all purchased from Merck Millipore and dissolved in dH_2O. Recombinant human TNF-α and TGF-β1 were purchased from R&D Systems (Abingdon, UK) and diluted in PBS and dH_2O containing 4 mM hydrochloric acid (HCL) and 1 mg/mL BSA respectively. Human IL-4 and IL-13 were purchased from Peprotec Ltd (London, UK) and dissolved in 1 mg/mL BSA. Human M-CSF recombinant protein was purchased from eBioscience and made up in PBS containing 1% BSA. Phorbol-12 myristate 13-acetate (PMA) was made up in DMSO and purchased from Merck Millipore.

2.1.3. Inhibitors

The PI3K inhibitor LY294002 (440202), mitogen-activated protein kinase 1 (MEK1)/ ERK1/2 signalling pathway inhibitor PD98059 (513000), p38 inhibitor SB203580 (559389) and pan-PKC inhibitor bisindolylmaleimide I (203290) were purchased from Merck Millipore and diluted in DMSO. The PPAR-γ inhibitor GW9662 was purchased from Cambridge Bioscience Ltd (Cambridge, UK) and diluted in DMSO.

2.1.4. siRNA

Plus smart pool siRNA for TREM-2, STAT-6, activating factor 2 (ATF2) and non-target control were purchased from Thermofisher (Pittsburgh PA, US). SMAD3 siRNA experiments used siRNA Silencer® Negative Control No. 1 siRNA (scrambled) or SMAD3 Duplex2 (Life Technologies, Paisley, UK).

2.2. Cell Culture

2.2.1. Heat Inactivation of Fetal Calf Serum

Fetal calf serum (FCS) (PAA) was inactivated by heating to 56°C in a water bath for 50 min before aliquoting into 50 mL Falcon tubes and storing at -20°C.

2.2.2. Cell Media

Cell culture media containing FCS was made by adding 0.45 µm of filtered pre-warmed (37°C) FCS to the media. After the addition of the FCS, all other solutions were added. Unless otherwise stated Roswell Park Memorial Institute medium (RPMI) 1640, Dulbecco's modified Eagle's medium (DMEM, with 4.5g/L glucose) and Iscove's modified Dulbecco's media (IMDM) media were complete i.e. contained 10% heat-inactivated FCS, 100 U/mL penicillin and 100 µg/mL streptomycin and 2 mM L-glutamine, all from PAA. The media was stored at 4°C and heated to 37°C before use.

2.2.3. Cells

Full details of the cells types used in this study and their maintenance requirements are detailed in Table 2.1.

Table 2.1: Primary cells and cell lines used. All cells were maintained at 37°C in the presence of 5% CO_2 in high humidity. P No. = passage number. All cell culture plastics were obtained from Fisher.

Cell type	Source	Media	Splitting procedure	P No.	For experimentation	Refs
THP-1	Peripheral blood of a 1 year old male with myeloid leukaemia. Purchased from ECACC*.	Complete RPMI.	1 in 2 every 3.5 days. Maintained between 2-7 x 10^5 cells/ml.	3-30	1 x 10^6 cells/ml, 1 mL media in 24-well plates 16 h before treatment. In some experiments cells were treated as above but in RPMI media without FCS.	(382)
RAW 264.7	Mouse leukemic monocyte macrophage cell line from a tumour induced by Abelson murine leukaemia virus. Purchased from ECACC*.	Complete DMEM.	1 in 5 - 1 in 10 every 3.5 days at 70-80% confluency using a scraper.	5-20	Seeded into 6-well plates until 70-80% confluency in DMEM media without FCS at least 24 h before experimentation.	(383)
HL60	Human myeloid cell line isolated from the peripheral blood of a 6-year-old Caucasian female with acute promyelocytic leukaemia. Purchased from ECACC*.	Complete RPMI .	1 in 25 every 3.5 days. Maintained between 1-9 x 10^5 cells/ml.	≤30	1 x 10^6 cells/ml, 1 mL media in 24-well plates 16 h before treatment.	(384).
A549	Human airway type II alveolar-like epithelial cells derived from a lung carcinoma from a 58-year old Caucasian male. Purchased from ECACC*.	Complete DMEM.	1 in 3 – 1 in 6 at 70-80% confluency using 0.25% trypsin/EDTA.	90-120.	Seeded into 6-well plates until 70-80% confluency, at least 24 h before experimentation.	(385).
3T3-L1	Murine fibroblast cell line. Purchased from ECACC*.	Complete DMEM.	1 in 50 at 70-80% confluency using 0.25% trypsin/EDTA.	≤13	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	(386)

SAECs	Human Small Airway Epithelial Cells. Purchased from Lonza (Slough, UK).	BulletKits basal media with SingleQuots growth factors (Lonza).	Maintained between 4 x 10^5 – 1 x 10^6 cells/mL when 70-80% confluent. Media changed every 2 days. Protocol detailed in section 2.2.6.	1-3	Seeded into 96 well plates and left until 70-80% confluency, at least 24 h before experimentation.	
16HBE	16HBE14o SV-40 transformed human malignant differentiated bronchial epithelial cell line. Obtained from Dr. Gruenert at The University of Vermont.	Complete MEM (Life Technologies).	1 in 30 every 3.5 days at 70-80% confluency using 0.25% trypsin/EDTA. Grown on fibronectin coated flasks (see Appendix Section 8.1.1).	11-33	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	*(387)*
ARPE-19	Human retinal epithelial cell line derived from the normal eyes of a 19-year-old male. Purchased from ATCC*.	Complete DMEM/F12 (Invitrogen).	1 in 10 every 7 days at 70-80% confluency using 0.25% trypsin/EDTA and media changed every 3.5 days.	≤20	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	(388).
NCI-H292	Human airway bronchial epithelial cells derived from a lymph node metastasis of a pulmonary mucoepidermoid carcinoma. Purchased from ATCC*.	Complete RPMI .	Passaged 1 in 30 every 3.5 days at 70-80% confluency using 0.25% trypsin/EDTA.	11-33	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	(389).
MIO-M1	The Moorfields/Institute of Ophthalmology-Müller-1 (MIO-M1) cell line is a spontaneously immortalised cell line isolated from the cornea of a 68 year old female. Obtained from Dr. A. Limb from the Institute of Ophthalmology at University College London.	Complete DMEM Glutamax media (Invitrogen).	1 in 5 every 7 days at 70-80% confluency using 0.25% trypsin/EDTA and media changed every 3.5 days.	≤33	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	(390)

CHME-5	Human fetal microglial cell line transfected with SV40 large T antigen. Obtained from Prof. Pierre Talbot, INRS-Institut Armand-Frappier (Canada).	Complete DMEM.	1 in 10 - 1 in 20 every 3 days at 70-80% confluency using 0.25% trypsin/EDTA.	15-25	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	[391] [392].
RGC-5	Murine neuronal precursor cell line obtained from Dr. Neeraj Agarwal from the University of North Texas (US).	Complete DMEM.	1 in 5 every 3 days at 70-80% confluency using 0.25% trypsin/EDTA.	9-13	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	[393]
N9	Murine microglial cell line. Obtained from Dr. Ji Ming Wang at The Center for Cancer Research, National Cancer Institute at Frederick (US).	Complete IMDM.	1 in 10 at 70-80% confluency using a cell scraper every 3-5 days.	≤20	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation. For oxygen glucose deprivation experimentation, N9 cells were maintained in DMEM with or without glucose and with 10% or 1% FCS as indicated.	[394].
SH-SY5Y	Human neuroblastoma cell line and were kindly donated by Prof. Marcus Rattray, University of Bradford (UK).	Complete DMEM.	1 in 10 every 3 days at 70-80% confluency using 0.25% trypsin/EDTA ensuring both suspension and adherent cells were maintained.	≤20	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation. In some experiments cells were maintained in DMEM containing 1% FCS.	[395].
Caco-2	Human colon adenocarcinoma cell line, purchased from ECACC*.	DMEM containing 10% FCS, 1% non-essential amino acids and 1 mM sodium pyruvate.	1 in 3 every 2-3 days at 70-80% confluency using 0.25% trypsin/EDTA.	50-70	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	[396]
UACC 1273	Isolated from the lymph node of a 54 year old male with malignant melanoma. Obtained from Dr. Antoni Ribas from University of California, Los Angeles (US).	Complete RPMI.	At 80% confluency with 0.25% trypsin/EDTA.	≤10	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	[397]

SK-MEL28	Isolated from the skin of a 51 year old male with malignant melanoma. Obtained from Dr. Antoni Ribas from University of California, Los Angeles (US).	Complete RPMI.	At 80% confluency with 0.25% trypsin/EDTA.	≤10	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	(398)
A2058	Isolated from the lymph node of a 43 year old male with malignant melanoma. Obtained from Dr. Antoni Ribas from University of California, Los Angeles (US).	Complete RPMI.	At 80% confluency with 0.25% trypsin/EDTA.	≤10	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	(399)
M202	Isolated from the lymph node of a patient with malignant melanoma. Obtained from Dr. Antoni Ribas from University of California, Los Angeles (US).	Complete RPMI.	At 80% confluency with 0.25% trypsin/EDTA.	≤10	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	(400)
Mel-501	Isolated from the lymph node of a patient with malignant melanoma Obtained from Dr. Antoni Ribas from University of California, Los Angeles (US).	Complete RPMI.	At 80% confluency with 0.25% trypsin/EDTA.	≤10	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	(401)
HUVEC	Pooled human umbilical vein endothelial cells. Purchased from TCS Cellworks (Buckingham, UK).	Large vessel endothelial cell media (plus **)	At 70-80% confluency with 0.25% trypsin/EDTA every 2 days Grown on fibronectin coated flasks (see Appendix Section 8.1.1).	2-4	Seeded into 6-well plates coated with fibronectin and left until 70-80% confluency, at least 24 h before experimentation.	(402).
MRC-5	Human lung fibroblast cell line. Purchased from ECACC*.	Complete DMEM media	1 in 4 every 3 days at 70-80% confluency using 0.25% trypsin/EDTA.	≤20	Seeded into 6-well plates and left until 70-80% confluency, at least 24 h before experimentation.	(403)

* European Collection of Cell Cultures (ECACC) (Salisbury, UK), American Type Culture Collection (ATCC).

** Supplemented with hydrocortisone, epidermal growth factor, fibroblast growth factor, Heparin, FBS (2% v/v) and 25 µg/mL gentamicin & 50 ng/mL amphotericin (TCS Cellworks)

2.2.4. Cell Counting and Viability

Trypan Blue solution (0.4%) (Sigma) was added to an aliquot of cells to assess cell viability, according to manufacturer's instructions. Viable cells do not allow the dye to permeate the membrane and therefore, cells that take up the blue dye were excluded from the cell count using light microscopy. Cells were counted under a light microscope using a Neubauer haemocytometer (Superior, Germany). 10 µL of cells were added to the haemocytometer chamber and the average number of cells per square counted. Each square corresponds to 0.1 µL volume, the following calculation was used to measure the number of cells in 1 mL:

$$\text{Number of cells (cells/mL)} = \text{Average count per section} \times \text{Dilution Factor} \times 10^4$$

2.2.5. Cell Passaging using 0.25% Trypsin/EDTA

The 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) solution (Sigma) was used for passaging specific adherent cell lines (Table 2.1). The media was removed and the cells washed in PBS followed by 5 mL of 0.25% trypsin/EDTA for up to 1 min (depending on cell type) then discarded. The cells were put back into the incubator (37°C) until the adherent cells had lifted from the base of the flask (no longer than 5 min). The cells were then resuspended in the correct media for the particular cell type (Table 2.1).

2.2.6. Cell Passaging of SAECs

The excess media was removed and the cells washed with 5 mL of HEPES-BSS (Lonza) before the addition of 2 mL of trypsin/EDTA solution (Lonza). Once the cells had lifted from the base of the flask, 4 mL of Trypsin Neutralising Solution (Lonza) was added and the cells centrifuged at 220 x g for 5 min. The supernatant was removed and the pellet resuspended in 2-3 mL of growth media. The media was replaced the day after cell seeding and every other day after that.

2.2.7. Freezing and Thawing Cells

Freezing cells: 5 x 10^6adherent or suspension cells, in logarithmic phase of growth, were centrifuged at 1200 rpm for 7 min. The cell pellet was resuspended in 1 mL of freezing media (1 mL of HYBRI-MAX DMSO (Sigma) plus 9 mL sterile filtered FCS). The cells were initially frozen in cryovials (Fisher) at -80°C, insulated in cotton wool to allow for gradual freezing. After at least 16 h the cryovials containing the cells were transferred to liquid nitrogen for long-term storage.

Thawing cells: Both suspension and adherent cells were defrosted in a 37°C water bath, and once defrosted, diluted in complete media specific for the cell type (Table 2.1) and centrifuged at 1200 rpm for 7 min. After centrifugation, the supernatant was removed and the cell pellet resuspended in fresh media in a T75 cell culture flask. The cells were placed in an incubator at 37 °C in the presence of 5% CO_2 in high humidity.

2.2.8. Autologous Serum and PBMC Isolation from Whole Blood

2.2.8.1. Ficoll Method

Ethical approval for the use of human blood in these experiments was obtained from the University of East Anglia's Faculty of Health Research Ethics Committee (Reference 2012/2013 – 25 HT). Healthy volunteers donated 20-100 mL of blood. The blood was collected in Falcon tubes containing 3.2% BioReagent Citrate Concentrated Solution (Sigma), at a ratio of 1:9 (citrate:blood). 25 mL of blood was layered on top of 15 mL Ficoll Paque Plus (GE Healthcare, Buckinghamshire, UK) and centrifuged at 400 x g for 35 min, with the brake off [404]. This caused the blood to separate into erythrocyte/granulocyte, Ficoll, plasma and PBMC layers (Figure 2.1). The top plasma layer was discarded and the PBMC layer collected into another tube and diluted to three times its volume in Hanks buffered salt solution (HBSS) and centrifuged at 60-100 x g for 10 min at 18-20˚C. The pelleted cells were resuspended in HBSS and centrifuged again at 60-100 x g for 10 min at 18-20˚C before resuspension in complete RPMI media. 8 x 10^6 PBMCs were seeded into each well of a 24-well plate and left for 1 h to adhere. The majority of monocytes adhered to the plate after this time, while all other cells (pooled lymphocyte fraction)

were removed and cells washed with PBS. Complete RPMI media (1 mL) was added to the monocytes and they were left in culture overnight before stimulation.

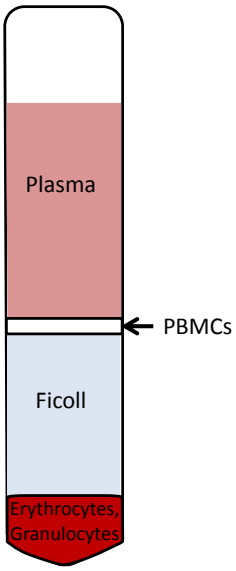

Figure 2.1: Isolation of PBMCs from whole blood. The PBMCs form a layer between the Percoll or Ficoll layer (either can be used) and the plasma layer. The cells with the highest density (erythrocytes and granulocytes) are found at the bottom of the tube, the least dense fraction at the top (plasma) and the Percoll/Ficoll layer in the middle.

2.2.8.2. Percoll Method

Whole blood was collected into sodium citrate tubes as detailed above (2.2.8.1) and centrifuged at 300 x g for 20 min. The solution at the bottom of the tube containing the blood cells was resuspended in HBSS up to the original volume of blood. This solution was gently layered over 10 mL 68% isotonic Percoll (GE Healthcare), containing 1 mL 10X PBS, 6.8 mL Percoll and 2.2 mL dH_2O. The tubes were centrifuged at 700 x g for 20 min with the brake off. After this time the PBMCs were removed and treated as above (2.2.8.1).

After isolation, PBMCs were either immediately processed, incubated with a differentiation inducer, e.g. M-CSF or left overnight before stimulation.

2.2.9. Peripheral Blood Monocyte and THP-1 Cell Differentiation

THP-1 monocytic cells were differentiated into macrophage-like cells in the presence of 100 nM PMA for 3 days [405, 406]. Primary monocytes were differentiated into monocyte derived macrophages with 50 ng/mL M-CSF for 7 days [407].

2.2.10. siRNA Transfections

THP-1 cells were seeded at 2 x 10^5 cells/mL into T75 flasks 48 h before electroporation. For transfection, 2 x 10^6 cells at a density of 3 - 4 x 10^5 cells/mL were centrifuged at 90 x g for 10 min and the pellets resuspended in 100 µL of RPMI 1640 (Life Technologies) (no FCS or pen/strep or L-glutamine) with or without 250 nM On Target Plus Smartpool siRNA (ATF-2, TREM-2 or STAT-6) or On Target Plus Non-Targeting Pool (Thermofisher Scientific, CO, US). The cells were electroporated in 0.2 cm electroporation cuvettes (Sigma) on programme V-001 on the Amaxa Nucleofector II Device (Lonza, Basel, Switzerland) and placed into 1.5 mL of RPMI media containing 10% FCS and pipetted into a 24-well plate. After 24 h the cells were centrifuged at 5000 rpm for 5 min and resuspended in 1 mL TRI Reagent (Invitrogen) or 1X sodium dodecyl sulfate (SDS) sample buffer (Invitrogen) for RNA or protein analysis (respectively). In some experiments, after 24 h the cells were stimulated with either TGF-β1 or IL-4 for up to 48 h as indicated in the figure legends.

2.2.11. SMAD3 KD

SMAD3 KD experiments were performed by Thomas Davies from The University of Cardiff in Dr. Dipak Ramji's research group. SMAD3 was knocked down using SMAD3 Duplex2 siRNA using Interferin (Polyplus, Nottingham, UK). THP-1 cells were seeded into 12-well plates at 2 x 10^5 cells/mL in 1 mL for real time polymerase chain reaction (RT-PCR) or 2.5 mL into 6-well plates at 1.25 x 10^6 cells for western blot analysis. Interferin was added to the cells for 4 h, 9 µL into the 12-well plate and 12 µL into the 6-well plate. Scrambled siRNA or SMAD3 Duplex2 siRNA were added to 100 µL (RT-PCR) or 250 µL (western blot) of RPMI (no penicillin, streptomycin or FCS) to make a concentration of 105 nM for 15-20 min and then added to the wells containing the THP-1 cells and incubated for 24 h resulting in a siRNA concentration of 9.55 nM. After 24 h, the cells were stimulated with TGF-β1 for 16 or 48 h. THP-1 cells were centrifuged at 150 x g for 5 min. The pellets were washed in PBS by centrifugation. Cell pellets were lysed in 50 µL radioimmunoprecipitation assay lysis buffer (Sigma) and a protease inhibitor cocktail (1 µL in 100 µL) (Sigma) for protein analysis and lysed on ice for 10 min. The cell debris was removed by centrifugation at 12,000 x g for 5 min and the supernatants

collected and stored at -80 °C until use. The pellets from the RT-PCR samples were resuspended in 0.75 mL TRIzol (Life Technologies, Paisley, UK) and incubated for 5 min.

THP-1 cells lysed in TRIzol were incubated with 200 µL chloroform, shaken vigorously for 15 sec then incubated at room temperature (RT) for 2-3 min. The rest of the steps were performed the same as the TRI Reagent protocol as described below (2.4.1). RNA was converted to complimentary DNA (cDNA) using Promega RT-PCR reagents (Promega, Southampton, UK). RT-PCR was performed as described below (2.4.5).

Protein samples were delivered on ice to the University of East Anglia and analysed by western blot analysis as described below (2.5.2).

2.2.12. Stimulation and Inhibitor Experiments

THP-1 cells were seeded at 1 x 10^6 cells/ml in 1 mL media in a 24-well plate, 16 h before experimentation. Cells were stimulated with 10 µg/mL LPS, 10 µg/mL PGN, 100 ng/mL Pam3Cys, 10 ng/mL TNF-α, 5 ng/mL TGF-β1, 10 ng/mL IL-4 or 10 ng/mL IL-13 for up to 7 days (time points indicated in figure legends). The following inhibitors were used to analyse cell signalling mechanisms: PKC inhibitor (bisindolylmaleimide I), p38 MAP kinase inhibitor (SB203580), MEK1/ERK1/2 signalling pathway inhibitor (PD98059), PI3K inhibitor (LY294002), or the PPAR-γ inhibitor (GW9662) (see figure legends for concentrations). The cells were pre-incubated with the inhibitor 30 min prior to addition of stimulus. Suspension cells were centrifuged at 2000 rpm for 5 min and the supernatant removed. The cell pellet was resuspended in 1 mL TRI Reagent (Invitrogen) for RNA isolation or 100 µL 1X Novex Tris-Glycine SDS sample buffer (diluted from 2X Tris-Glycine SDS in PBS) (Life Technologies) for Western blotting (see Section: 2.5). For adherent cells, the media was removed and the cells lysed in 1 mL TRI Reagent for RNA isolation or 200 µL 1X Novex Tris-Glycine SDS sample buffer. For detection of secreted proteins, the supernatants were collected on ice and stored at -80°C and analysed by western blotting.

2.3. MTS Assay

The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) was used to measure cell viability. Cells were seeded into a 96-well plate at the cell density required for the cell type and either left to adhere (adherent cells) for at least 16 h or immediately exposed to the stimulus (suspension cells) for various times as indicated in figure legends. Once the experiment had finished, 10 µL of CellTiter 96® AQueous One Solution was added to each well and left for 1-8 h depending on cell type at 37˚C. The CellTiter 96® solution contains 3-(4,5-dimethylthiazol-2-yl)- 5-(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate (electron coupling reagent). In living cells, the reducing agent NADPH, reduces MTS into formazan (absorbance 490 nm), a water soluble dye. Increased cell viability was recognised by increased absorbance at 490 nm, due to increased metabolism of the substrate.

2.4. Analysis of mRNA Expression

2.4.1. RNA Isolation

For RNA isolation, 0.5 - 1 x 10^6 cells were resuspended in 1 mL TRI Reagent Solution (Life Technologies) and incubated at RT for 5 min [408]. At this stage the samples were either stored at -80°C for up to three weeks or used immediately for RNA extraction. For RNA extraction, 100 µL of bromo-chloropropane (Sigma) was added to the TRI Reagent sample and the tubes shaken vigorously for 10 sec. The samples were then incubated for 10 min at RT and centrifuged (12,000 x g, 20 min, 4°C), forming layers in the samples. The upper aqueous phase was removed and placed into a new tube containing 500 µL 2-propanol (Sigma) and vortexed for 10 sec. The samples were incubated at RT for 10 min before centrifuging at 12,000 x g for 15 min at 4°C. The supernatant was removed and the pellet resuspended in 70% molecular grade ethanol (Sigma). The samples were then gently vortexed for 5 sec and centrifuged at 12,000 x g for 10 min at 4°C. After centrifugation, the ethanol was removed (as much as possible without disturbing the pellet) leaving the rest to evaporate. Once all the ethanol had evaporated the RNA was resuspended in 10-20 µL distilled RNase, DNase free H_2O (Fisher) depending on quantity of RNA expected and put into the -80°C freezer overnight before the next step. Alternatively, the RNA could be stored at -80°C for at least one year.

2.4.2. RNA Quantification and Reverse Transcription

Total RNA was quantified using the Nanodrop 1000 Spectrophotometer (Lab Tech, Uckfield, UK) and diluted with distilled RNase, DNase free dH_2O to 100-200 ng/µL. The Nanodrop measured the quantity of RNA using the Beer-Lambert Law:

OD = εCb

OD = absorbance at 260 nm

ε = extinction coefficient

C = concentration

b = path length

The extinction coefficient for RNA is 40 µg/mL. With this information and the absorbance of the sample at 260 nm the Nanodrop calculates the concentration of RNA in the sample in ng per µL. To ensure high quality of RNA, the ratio of absorbance at 260/280 and 260/320 shown the degree of contamination of the sample. Values between 1.8 – 2 indicate that the sample is pure and free of contamination.

For cell expression studies, samples were diluted to 150 ng/µL. 100-200 ng (150 ng for cell expression studies) of RNA was added to 0.2 mL eppendorf tubes up to a volume of 4.5 µL in distilled RNase, DNase free dH_2O. RNA was converted to cDNA by adding 5.5 µL of RT Master Mix (Life Technologies, see Appendix Section 8.1.2) to each sample. All the samples were placed in a PTC-100 thermocycler (Bio-Rad, Herts, UK) and incubated for 10 min at 21°C, 15 min at 42°C, 5 min at 99°C and 5 min at 4°C. After this time, the samples were diluted with 15-20 µL distilled RNase, DNase free dH_2O and used immediately or frozen for future use in quantitative RT-PCR experiments.

2.4.3. cDNA Direct from Cell Culture

Due to the limited number of passages and slow proliferation of the SAECs, the Cells-cDNA II kit (Life Technologies) was used to obtain cDNA directly from cell extracts so that fewer cells could be used to obtain similar amounts of cDNA. The cells were washed twice in ice cold PBS, then resuspended in 50 µL of Cells-to

cDNA lysis buffer and incubated for 10 min at 75°C. At this point, samples could be frozen and the protocol continued at a later date. Once samples had cooled (or thawed) to RT, 1 µL of DNase I was added to each sample and incubated for 15 min at 37°C then a further 5 min at 75°C. 8 µL from each sample was transferred into a new tube and the remaining sample stored at -20°C. At this point, 1 µL of Random Hexamers (pd(N)6) and 3 µL of Deoxynucleotide Triphosphates (dNTPs) were added and the sample incubated for 5 min at 70°C. The samples were chilled on ice before the addition of: 2 µL of 10X RT buffer, 2 µL 0.1 M dithiothreitol (DTT), 3 µL ddH_20, 0.5 µL Moloney Murine Leukaemia Virus reverse transcriptase and 1 µL RNaseOUT. The samples were then incubated for 50 min at 37°C, followed by 15 min at 75°C. After this time the samples were diluted with 30 µL of double distilled RNase, DNase free dH_2O. The cDNA samples were now used for quantitative real time-polymerase chain reaction (qRT-PCR) (see below) or stored at -20°C until required (no longer than 1 month).

2.4.4. Primer Synthesis

Primers were designed for specific exon-spanning regions using NCBI primer BLAST to ensure the primer would not bind to cDNA other than the gene of interest (Table 2.2).

Table 2.2: **Primer sequences:** Unless otherwise stated, all custom designed primers were purchased from Life Technologies. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Gene	**Primer Sequence**
Human GAPDH	Forward: 5’ AAC AGC CTC AAG ATC ATC AGC A 3’ Reverse: 5’ TGC TAA GCA GTT GGT GGT GC 3’
Mouse GAPDH	Forward: 5’ AGC TTG TCA TCA ACG GGA AG 3’ Reverse: 5’ TTT GAT GTT AGT GGG GTC TCG 3’
Human TREM-2	Forward: 5’ TCT GAG AGC TTC GAG GAT GC 3’ Reverse: 5’ GGG GAT TTC TCC TTC CAA GA 3’
Human TREM-2 (used for SMAD3 KD study)	Forward: 5’ TCT GAG AGC TTC GAG GAT GC 3’ Reverse: 5’ GGG GAT TTC TCC TTC CAA GA 3’
Mouse TREM-2	Forward: 5’ TGG GAC CTC TCC ACC AGT T 3’ Reverse: 5’ GTG GTG TTG AGG GCT TGG 3’

Human IL-1β	Forward: 5' GGA CAA GCT GAG GAA GAT GC 3' Reverse: 5' TCG TTA TCC CAT GTG TCG AA 3'
Human STAT-6	Forward: 5' GTT CCG CCA CTT GCC AAT G 3' Reverse: 5' TGG ATC TCC CCT ACT CGG TG 3'
Human MMP-1	Forward: 5' GCT AAC CTT TGA TGC TAT AAC TAC GA 3' Reverse: 5' TTT GTG CGC ATG TAG AAT CTG 3'
Human IL-8	Pre-designed by Qiagen
Human ATF2	Forward: 5' TTT CCT CCA GGG GTG CTT TG 3' Reverse: 5' GCA GTC CTT TCT CAA GTT TCC 3'
Human SMAD3	Forward: 5' GAG CAA TAT TCC AGA GAC CCC ACC C 3' Reverse: 5' TTT GGA GAA CCT GCG TCC ATG CT 3'
Human GAPDH (used for SMAD3 KD study)	Forward; 5' CTT TTG CGT CGC CAG CCG AG 3' Reverse: 5' GCC CAA TAC GAC CAA ATC CGT TGA CT 3'
Mouse IL-1β	Forward: 5' TGT AAT GAA AGA CGG CAC ACC 3' Reverse: 5' TCT TCT TTG GGT ATT GCT TGG 3'
Mouse TGF-β1	Forward: 5' TGG AGC AAC ATG TGG AAC TC 3' Reverse: 5' CAG CAG CCG GTT ACC AAG 3'

2.4.5. qRT-PCR

For qRT-PCR [409], 5 µL of cDNA and 15 µL PCR Mix (see Appendix Section 8.1.2) were transferred into 200 µL PCR tubes. Standards made using equal quantities of cDNA from each sample, using a one in two serial dilution in distilled RNase, DNase free dH_2O to produce five standards. The samples were added to tubes containing 15 µL PCR Mix (see Appendix Section 8.1.2). A separate tube was set up with 5 µL distilled RNase, DNase free dH_2O and 15 µL PCR Mix as a No Template Control (NTC) to check for DNA or RNA contamination. The samples were placed in the Qiagen Rotor-Gene Q machine for one cycle at 95°C for 120 sec followed by 40 cycles where each cycle ran for 15 sec at 95°C and 40 sec at 40°C. The samples and standards for the gene of interest were normalised to a control gene (GAPDH) for each sample/standard. Results were analysed using the Rotor-Gene 600 Series Software 1.7. Relative concentrations were produced using the standard curve method. However, since the concentration of the gene of interest in the standards is unknown, this was based on the knowledge that standard two has half the

concentration of the gene of interest than standard one and so on. In addition, a melt analysis was performed to analyse the quality of the PCR reaction and the effectiveness of the primers.

2.5. Western Blotting

2.5.1. Sample Preparation

Whole Cell Extracts: For whole cell extracts, 1 x 10^6 suspension cells were centrifuged at 2000 rpm for 5 min and resuspended in 100 µL 1X Novex Tris-Glycine SDS (see Appendix Section 8.1.3) (Life Technologies). For adherent cells, 100 or 200 µL 1X Tris-Glycine SDS was added to each well in a 24-well plate or 6-well respectively and the sample placed into eppendorf tubes. The samples were boiled for 5 min, then pulse centrifuged. At this stage samples could be stored at -80°C for future use. Protein concentration was measured using absorbance at 280 nm with the Nanodrop 1000 Spectrophotometer using the Beer Lambert Law as detained above (Section 2.4.2) (extinction coefficient 1 mg/mL). The amount of sample loaded onto the gel was altered, depending on protein concentration so each sample was loaded onto the gel with equal concentration. This was particularly important for the cell expression studies. In addition, for the cell expression studies, PMA-differentiated THP-1 cells were run on each gel so that the expression in each cell type could be compared to the same control.

Nuclear and Cytosolic Extracts: For nuclear and cytosolic extracts, 5 x 106 cells were centrifuged at 1200 rpm for 7 min at 4°C and resuspended in 1 mL ice-cold PBS. The cells were then centrifuged at 5000 rpm for 1 min at 4°C and resuspended in 50 µL Buffer A (see Appendix Section 8.1.3) and incubated on ice. After 5 min the solution was centrifuged at 13,000 rpm for 8 sec and the supernatant collected as the cytosolic fraction and stored on ice until the end of the procedure. The pellet was resuspended in 100 µL Buffer A, and centrifuged at 13,000 rpm for 8 sec. The supernatant was discarded and the pellet was resuspended in 50 µL Buffer B (see Appendix Section 8.1.3). The pellet was sonicated for 10 sec, using a Heilscher UP50H Ultrasonic Processor (Teltow, Germany), followed by centrifugation at 13,000 rpm for 8 sec. The supernatant was collected and pipetted into a new eppendorf tube, and this was the nuclear fraction. The nuclear and cytosolic fractions were snap-frozen using dry ice and ethanol and stored at -80°C.

Two different methods were used for western blotting, the Bio-Rad system which used polyacrylamide gels made in the lab and semi-dry transfer and the Novex system (Invitrogen, Paisley, UK) that used pre-cast gels and a wet transfer system.

2.5.2. Gel Electrophoresis: Bio-Rad

To make 10% polyacrylamide gels, 30% acrylamide, dH_2O, tetramethylethylenediamine (TEMED) and 10% ammonium persulfate (APS) (in dH_2O) (all purchased from Fisher) were all added to the resolving gel stock (see Appendix Section 8.1.3). The solution was added to clean gel casting glass plates (Bio-Rad), leaving a gap at the top for the stacking gel. The stacking gel was made up by adding 30% acrylamide, dH_2O, TEMED and 10% APS (in dH_2O) to the stacking gel stock (see Appendix Section 8.1.3). This solution was added to the set resolving gel and a 10 or 15-well comb inserted and left to set for up to 30 min. The gels were stored at 4°C for up to 3 days.

One or two gels were placed into the Bio-Rad mini protein gel rig and filled with running buffer (see Appendix Section 8.1.3). For cell supernatants, 6.5 µL of the cell culture supernatant was added to 2.5 µL 1X lithium dodecyl sulfate (LDS) sample buffer (see Appendix Section 8.1.3) (Life Technologies) and 1 µL 10X NuPAGE Reducing Agent (Life Technologies). For cell lysates, 10X NuPAGE Reducing Agent was added to each sample at a ratio of 1:9 (reducing agent: sample). All samples containing reducing agent were boiled for 5 min and pulse centrifuged before loading onto the gel. Gel loading varied depending on cell type and 5 µL of Pre-stained broad range SDS PAGE Standards (Bio-Rad) was used as a molecular weight marker. Electrophoresis was performed at 150 V for 1 h [410].

2.5.3. Gel Transfer: Bio-Rad

The PVDF (polyvinylidene difluoride) membranes (Bio-Rad) were cut to the same size as the gel and washed in methanol (Fisher) for 30 sec to activate the membrane. The membrane was then rinsed in dH_20 and soaked in 1X transfer buffer (see Appendix Section 8.1.3) for at least 15 min on a shaker. Prior to transfer, blot pads (Life Technologies) were soaked in 1X transfer buffer, compressing the

pads in the buffer to ensure there were no bubbles. When electrophoresis was complete, the casts were opened so that the gel remained on the back glass plate. For transfer of one gel (see Figure 2.2A), three pieces of soaked filter paper were placed on top of the gel and the membrane placed underneath the gel, followed by three more pieces of filter paper under that. The gel/membrane stack was placed onto the semi-dry blotter (Sigma) inside two pre-soaked blot pads, with the gel on top, nearest the cathode.

To transfer two gels (see Figure 2.2B), as with one gel, each gel was opened leaving the gel on the small back pate. There were nine pieces of filter paper on either side of the stack touching the blot pad and three pieces separating the two gels and membranes. As with one gel, the membranes were closer to the anode and the gels closer to the cathode. Transfers were run at 15 V for 45 min.

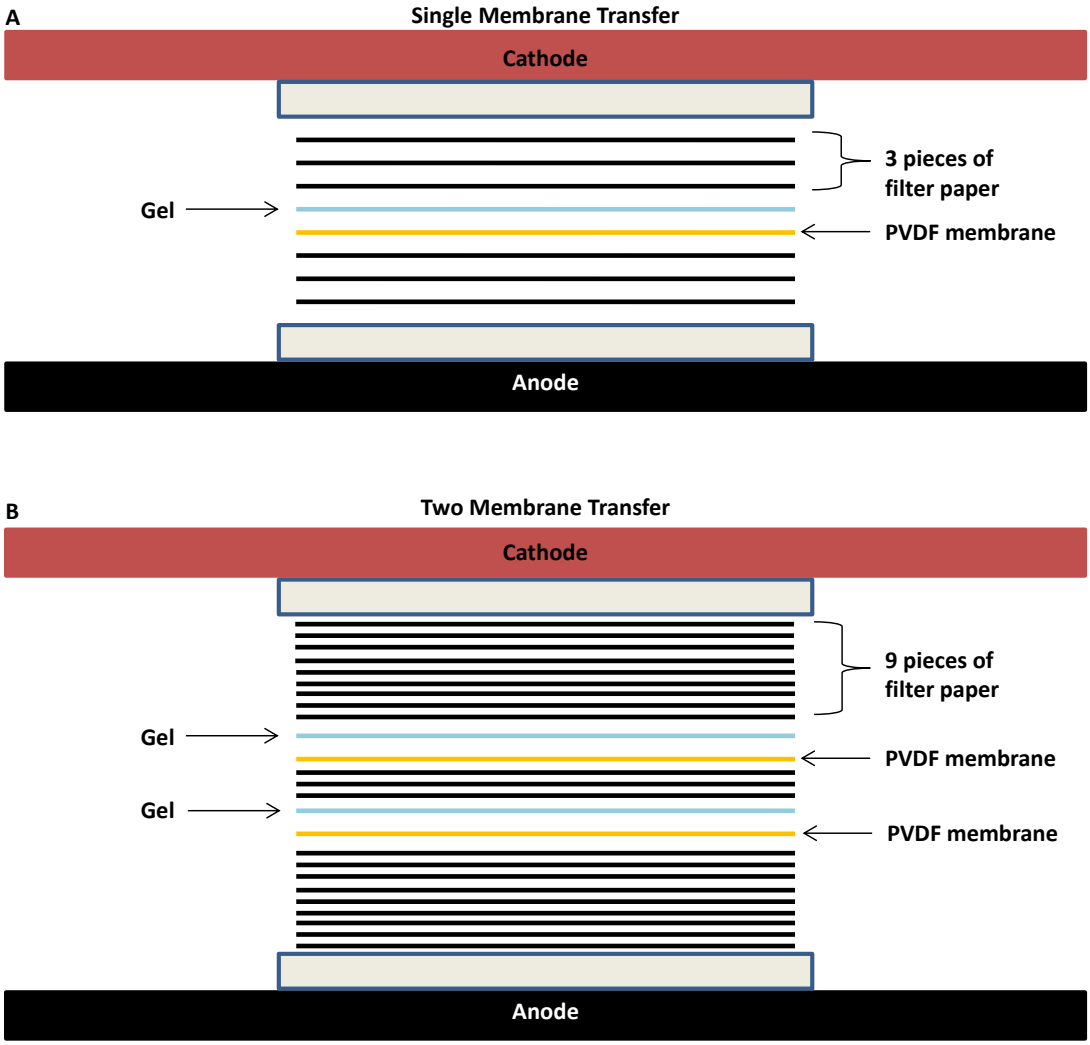

Figure 2.2: Semi-dry blotting system: A) Transfer of proteins from one gel to one membrane. B) Transfer of two gels to two membranes.

2.5.4. Gel Electrophoresis: Novex

A pre-cast Novex 4-12% Bis-Tris Gel (Life Technologies) was placed into the Novex X-cell Sure-lock gel rig (Life Technologies) and the surrounding areas were filled with 1X MOPS or MES running buffer (see Appendix Section 8.1.3) (Invitrogen). Sample loading and preparation was the same as the Bio-Rad gel electrophoresis (Section 2.5). Gel electrophoresis was performed at 150 V for 1 hr.

2.5.5. Gel Transfer: Novex

The PVDF membrane and blot pads were prepared as described above (Section 2.5.2). Following electrophoresis, the foot of the gel was cut and the gel casing opened, leaving the gel on the back plate. The soaked filter paper was placed on top of the gel and the wells and foot of the gel trimmed to fit the filter paper. The membrane and filter paper were transferred to a glass plate with the filter paper on the bottom and gel on top. A piece of PVDF membrane soaked in 1X transfer buffer (see Appendix Section 8.1.3) was added on top of the gel followed by another piece of soaked filter paper. Two pre-soaked blot pads were added to the gel rig and the gel/membrane assembly placed on top with the gel closest to the cathode. The remaining pads were used to fill the gel rig and the rig locked in place so transfer could begin. The surrounding areas were filled with 1X transfer buffer and the transfer run at 30 V for 1 h.

2.5.6. Membrane Blocking and Staining Procedure

The membrane was incubated in blocking solution (see Appendix Section 8.1.3) for 1 h on a shaker (or left overnight at 4˚C). Primary antibodies (see Table 2.3) were made up in blocking solution and placed into 50 mL Falcon tubes, with the membrane facing inwards, then left on a rotator for 1 h at RT or overnight at 4˚C. After this time, the membrane was washed briefly in 1X Tris-Buffered Saline with Tween 20 (TBST) (see Appendix Section 8.1.3), then washed three times in blocker followed by three washes in 1X TBST (5 min each). The membrane was then incubated with secondary antibody (in blocker) (see Table 2.3) in a Falcon tube on the rotator for 30 min. After incubation, membranes were washed as before (three times in blocker, three times in 1X TBST). After washing, the excess TBST was removed by blotting with filter paper and the Amersham ECL prime reagent (GE Healthcare) added onto the membrane for 5 min. The membrane was either immediately viewed on the G:BOX SynGene imager using the GeneTools and GeneSnap Software or placed in a RPN 18 x 24 cm Amersham autoradiography cassette (G E Healthcare) containing a 12.7 x 17.8 cm CL-XPosure Film (Fisher) and left for up to 5 min depending on the strength of the protein band expected. With this detection method, the film was developed using an X-Ograph X4 imager (X-Ograph, Gloucestershire, UK). β-actin was used to control for protein

loading in whole cell extracts and β-actin and histone H3 for cytosolic and nuclear fractions (respectively) in nuclear and cytosolic extracts.

Table 2.3: Antibodies used for western blot analysis or immunofluorescence

Antibody	Target species	Host Species	Concentration	Type	Company
TREM-2	Human	Goat	0.2 µg/mL (WB), 10 µg/mL (IF)	Polyclonal	R&D Systems
TREM-2	Mouse	Sheep	10 µg/mL (IF)	Polyclonal	R&D Systems
SMAD3	Human	Rabbit	1:1000	Monoclonal	New England Biolabs
Phosphorylated p38-MAPK (Thr180/Tyr182)	Human	Rabbit	1:1000	Monoclonal	New England Biolabs
p38-MAPK	Human	Mouse	1:1000	Monoclonal	New England Biolabs
IBA-1	Human	Goat	3.33 µg/mL (IF)	Polyclonal	Abcam (Cambridge, UK)
Histone H3	Human	Rabbit	1:5000	Polyclonal	Abcam
Beta-actin	Multiple	Mouse	1:500,000	Monoclonal	Sigma
Beta-COP	Mouse and Monkey	Rabbit	1:200	Polyclonal	In house- from Tom Wileman (UEA) [411]
MMP-1	Human	Rabbit	1:1500	Polyclonal	In house- from Ian Clarke (UEA) [412]
Alexa 594	Sheep	Donkey	1:100	Polyclonal	Stratech (Suffolk, UK)
Alexa 488	Goat	Rabbit	1:200	Polyclonal	Invitrogen
HRP	Mouse	Goat	1:5000	Polyclonal	Dako (Cambridge, UK)
HRP	Goat	Donkey	1:1000	Polyclonal	Santa Cruz Biotechnology (Heidelberg, Germany)
HRP	Rabbit	Goat	1:1000	Polyclonal	Santa Cruz Biotechnology
IgG Control	Goat	Goat	Same as test antibody	Whole antibody	Sigma

Abbreviations: WB; Western Blot. IF; Immunofluorescence.

2.5.7. Re-probing of Membranes

Membranes were re-probed for β-actin to check for protein loading. The membranes were stripped using 20 mL of 1X Reblot Plus Strong Solution (Millipore) (see Appendix Section 8.1.3) by incubation for 30 min on a shaker. After rinsing in blocker, the membrane was washed three times in blocker and left for 5 min on a shaker between washes. After this step the membrane could be re-blotted by following the protocol above starting at the step with the addition of a primary antibody.

Alternatively, the membrane was stripped with stripping solution made 'in house' (see Appendix Section 8.1.3). The membrane was incubated in the shaker for 10 min with the 'in house' stripping solution and then replaced with fresh stripping solution for a further 10 min. The membrane was washed twice in PBS for 10 min and twice in TBST for 5 min. The membrane was incubated on the shaker in blocker for 30 min before adding the primary antibody from here the procedure continued as described previously.

2.5.8. Densitometry

In some cases, densitometry was used to represent changes in protein expression observed by western blot analysis. Bands were measured using gel analysis software in Image J. Densitometry values used in Section 3.3.2 for protein expression in different cell types used PMA-differentiated THP-1 cells as a reference cell line for TREM-2 expression and divided the mean densitometry values of each cell line by that of the PMA-differentiated THP-1 cells to get values of mean fold change relative to PMA-differentiated THP-1 cells ± SEM.

2.6. Immunofluorescence

2.6.1. Cells

Adherent cells were grown on 22 mm sterile cover slips (Fisher) in 6-well cell culture plates (Fisher) at various seeding densities and durations depending on cell type.

Suspension cells were added to BioCoat 12mm poly-L-lysine coated coverslips (Scientific Laboratory Supplies, Hessle, UK) and left to adhere overnight. Following treatment, the media was removed from the wells and the cells washed three times in PBS then fixed with 4% PFA (see Appendix Section 8.1.4) for 30 min at RT. The cells were washed three times for 5 min in PBS. To permeabilize the cells, 0.1% Triton X-100 (Fisher) in PBS was added for 15 min at RT. The cells were then washed with 0.5 % BSA in PBS at least six times on a plate shaker at low speed. Alternatively, the cells were washed in PBS when using the donkey anti-sheep antibody, as sheep is too similar to bovine and therefore all washes and antibody dilutions were in PBS. To reduce non-specific binding of the secondary antibody the cells were incubated in the serum of secondary antibody host species, 10% in PBS for 1 h at RT. The cells were washed again as before, six times with 0.5% BSA in PBS. Primary antibodies (see Table 2.3) were diluted in a 1% BSA in PBS and 100 µL placed over the coverslip, covered with a plastic cover to prevent evaporation and incubated overnight at 4˚C. After 16 h the cells were washed in PBS as previously described, then incubated with the fluorescence-coupled secondary antibody (see Table 2.3), made up in 0.5% BSA in PBS. The cells were incubated with 100 µL of secondary antibody for 1 h at RT in the dark [413]. After this time, the cells were washed as described previously in PBS before the addition of 100 µL DAPI stain (1:400 in PBS, purchased from Sigma) for 10 min. The cells were washed again three times in PBS followed by one wash in water and mounted on a glass microscope slide using Hydromount mounting medium (National Diagnostics, Hessle, UK). For confocal microscopy, the slides were mounted with Citifluor AF1 (Citifluor Ltd, London, UK), which reduced photo-bleaching of the samples when used for long periods of time on the confocal microscope. Clear nail varnish was applied around the edges of the slide to keep the slide in place and preserve the sample. The cells were mounted facing downwards placing mounting medium onto the glass slide and slowly placing the coverslip onto it, ensuring there were no bubbles, and left to dry. Slides were imaged using Leica Microsystems CMS GmbH Fluorescence Microscope and Leica Application Suite software (Leica, Mannheim, Germany) or a Zeiss LSM510 META laser scanning confocal microscope (Carl Zeiss Ltd, Cambridge, UK) and analysed using Velocity 3D Image Analysis software (PerkinElmer, Massachusetts, US).

2.6.2. Human Organotypic Retinal Culture Sectioning, Oxygen Glucose Deprivation and Immunofluorescence

Ethical approval for the use of human tissue in these experiments was obtained from the University of East Anglia's Faculty of Health Research Ethics Committee (Reference 2012/2013 – 25 HT). Eye tissue was obtained from the East Anglian Eye Bank. The tissue was obtained and processed by Dr. Julie Sanderson's laboratory. The eyes were transported to the University of East Anglia (UEA) in EMEM (Sigma) containing 50 µg/mL gentamycin (Sigma), 10,000 U/mL penicillin G, 10,000 µg/mL streptomycin sulphate and 25 µg/mL amphotericin B (Life Technologies). The human organotypic retinal culture (HORC) tissue was dissected as previously described [(414)]. The eye tissue was cut between the ciliary body and the sclera (10 mm) and the tissue cut around the circumference of the globe under the ciliary body at the *ora serrate*. The HORC tissue was removed by rotating the globe and the neural retina removed by making a cut at the optic nerve head. The neural retina was put into a sterile petri-dish containing DMEM/HamF12 (Life Technologies) containing 2 mM L-glutamine and 50 µg/mL gentamycin (complete DMEM/HamF12). The vitreous humour was removed from the HORC tissue and discarded. The HORC tissue was cut into sections using a 4 mm diameter micro-dissecting trephine (Biomedical Research Instruments, MD, USA). The sections were placed into individual petri-dishes containing serum free complete DMEM/HamF12 media. The sections were incubated for 1 h before experimentation.

At the start of the experiment the media was changed to either control or glucose free DMEM (serum free) (Life Technologies). Immediately after the media change, the HORCs were placed into the modular incubator chamber (Billups-Rothenberg Inc) (Wolf Laboratories Limited, York, UK) and flushed through with N_2 containing 5% CO_2 for 10 min to remove any remaining oxygen. The chamber was sealed and the oxygen glucose deprivation (OGD) and control HORCs were placed back into the 37°C (times indicated in figures).

After treatment the HORCs were fixed in 4% PFA for 24 h at 4°C. The tissue was then immersed in 30% sucrose in PBS for 24 h at 4°C. The tissue was removed and mounted vertically in optimum cutting temperature (OCT) blocks followed by freezing using dry ice. Retinal tissue was sectioned into 13 µm slices using the Bright OTF 5000 cryostat) (Bright Instruments, Huntington, UK) and placed onto slides (Sigma) coated with 3-aminopropyltriethoxysilane (Sigma) to adhere and

stored at -20°C until use. For immunostaining [414], retinal tissue mounted slides were first washed in PBS three times for 10 min then incubated in blocking solution (see Appendix Section 8.1.4) for 90 min. After this time, the excess moisture from around the sections were removed before incubation with 50 µL primary antibody (in blocking solution without Triton X, Table 2.3) overnight at 4°C, covered in plastic to prevent evaporation. The sections were then washed three times in PBS for 5 min and the secondary antibody (in blocking solution without Triton X) added for 2 h at RT in the dark. The cells were washed again, three times in PBS (5 min each) then incubated with the nuclear stain DAPI (1:100 in PBS) for 10 min at RT in the dark. The sections were washed for the final time (three times in PBS for 5 min each), mounted onto a coverslip using Hydromount and left in the dark at RT overnight. The slides were viewed using Leica Microsystems CMS GmbH Fluorescence Microscope and Leica Application Suite software.

2.6.3. Immunostaining of Lung Tissue

Ethical approval for the use of human lung tissue in these experiments was obtained from the University of East Anglia's Faculty of Health Research Ethics Committee (Reference 2012/2013 – 25 HT). The paraffin embedded lung tissue was obtained from the Norwich and Norfolk Human Tissue Bank. Post-mortem tissue samples were obtained from healthy, asthmatic or COPD patients who had given authorised consent for research. Patient information was obtained in a linked anonymised way. Paraffin embedded blocks were cut into 4 µm sections using a Spencer 820 microtome (American Optical). Surgipath Sta-On Tissue Section Adhesive solution (diluted in water 10 mL in 1 L) (Leica Biosystems, Milton Keynes UK) was put on a glass slide and the sections placed on top, the glass slides were then briefly heated to 60°C to flatten the sections. The excess solution was removed using tissue and the slides left to dry at RT overnight. The sections were rehydrated by immersing the sections in xylene (Sigma) 2 x for 5 min and then in a decreasing ethanol (Sigma) gradient (5 min in 100%, 3 min in 90%, 2 min in 75%, 1 min in 50%). After the ethanol gradient, the sections were immersed in distilled water for 5 min.

The antigen retrieval step was optimised to analyse which buffer (EDTA or citric acid) resulted in the highest specific antibody binding. This optimisation found that the citric acid buffer (see Appendix Section 8.1.4) was the best buffer for antigen

retrieval, which took place in a water bath at 85°C for 20 min followed by 20 min to allow the sections to cool to prevent the sections drying out in the next step. To quench endogenous peroxidase activity, this study used dH_2O_2. 100 µL 0.3% dH_2O_2 (in methanol) was added to the sections and covered with plastic to prevent evaporation for 30 min. After this time, the cells were washed three times in 1X immuno TBST (see Appendix Section 8.1.4) for 5 min each on a shaker at low speed. The sections were then incubated with 2.5 % horse serum (included in the ImmPRESS Anti-Goat Ig (peroxidase) Polymer Detection Kit (Vector Laboratories, Peterborough, UK) for 1 h at RT. 10 µg/mL TREM-2 primary antibody (see Table 2.3) or goat IgG isotype control (see Table 2.3) were made up in 1.5% horse serum in 1X TBS (see Appendix Section 8.1.4). Excess 2.5% horse serum was removed from the sections using tissue and 100 µL TREM-2 antibody or isotype control added to the sections, covered in plastic and incubated overnight at 4°C.

The sections were washed six times for 5 min each in 1X immuno TBST on a low speed shaker. The sections were then incubated in the IMPRESS REAGENT anti-goat IgG provided by the ImmPRESS Anti-Goat Ig (peroxidase) Polymer Detection Kit for 30 min at RT. After this time, the ImmPACT NovaRED Peroxidase Substrate (Vector Laboratories) (see Appendix Section 8.1.4) was added for 15 min. The sections were then washed in distilled water for 5 min and then mounted using Hydromount and left to dry at 4°C overnight. The images were visualised using the L Microscope Leica DMR and Nikon digital sight DSi1 microscope camera and analysed with the NIS elements Software. The presence or absence of structures within the lungs and TREM-2 positive cells were confirmed by Dr. Mark Wilkinson, a pathologist at Norfolk and Norwich Hospital.

2.7. Statistics

qRT-PCR data and MTS data were analysed using one way ANOVA followed by Dunnett's or Bonferroni post hoc tests as indicated in figure legends. When only two samples were analysed and the samples were normally distributed, a two-sample student's t-test was performed. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. Values are expressed as mean ± SEM where appropriate. GraphPad Prism 5 (GraphPad Software Inc, CA, USA) and SPSS Statistics 22 (IBM Software, Portsmouth, UK) were used for the statistical tests. See figure legend for experimental replicates.

3. Characterisation of TREM-2 Expression in Cells and Tissues

Acknowledgements

Andy Osborne, a PhD student from Dr. Sanderson's laboratory (School of Pharmacy, UEA), performed the experiment and provided the image for Figure 3.9. He also set up the OGD experiment in HORC tissue in Figure 3.10 and Figure 3.11.

3.1. Introduction

TREM-2, or Triggering receptor expressed on myeloid cells – 2, as the name suggests, is largely expressed in myeloid cells, including dendritic cells, macrophages, osteoclasts and microglia [240, 243, 260, 338]. TREM-2 is a glycosylated transmembrane receptor protein, which has been confirmed by removal of N- and O- linked glycosylation in COS7 cells transfected with TREM-2 and in dendritic cells [278, 298]. The glycosylated forms of TREM-2 appear between 28 and 40 kDa by western blot analysis [278, 298, 359]. In myeloid cells, TREM-2 has mainly been studied on the cell surface due to its known functions in phagocytosis and suppression of TLR-mediated signalling [81, 240]. However, TREM-2 is also known to be expressed intracellularly, and is continuously transported to and from intracellular stores to the plasma membrane, though its intracellular functions are unknown [264].

In addition to its expression in myeloid cells, TREM-2 expression has also been observed in a small number of non-myeloid cell types including epithelial cells of the genitourinary tract and hepatic endothelial cells [241, 257]. The expression of TREM-2 in these non-myeloid cell types suggests that TREM-2 may also be expressed on other non-myeloid cells. Understanding the range of cells and tissues that express TREM-2 will aid the discovery of new functions of TREM-2 in health and disease. TREM-2 expression is increased in diseases of the lung including COPD and allergy, in cerebral ischaemia and in infections of the eye [242, 360, 367, 368]. The mechanisms of TREM-2 induction in these conditions are unknown and whether non-myeloid cell types contribute to the change in TREM-2 expression in these conditions is yet to be investigated. Understanding the cell types that express TREM-2 and the localisation of this expression may lead to the discovery of previously unknown functions of TREM-2 in health and disease.

3.2. Aims

The aims of this study were to:

- Characterise the expression and localisation of TREM-2 in myeloid cells
- Screen myeloid and non-myeloid cells for TREM-2 expression
- Investigate the expression of TREM-2 in the human retina and lung in health and disease states

3.3. Results

3.3.1. TREM-2 Protein Expression in Myeloid Cells

The first aim of this study was to confirm TREM-2 expression in myeloid cells. The expression of TREM-2 in macrophages and microglial cells has been widely documented and therefore these cell types were chosen to validate TREM-2 expression [81, 149, 240, 241, 261, 264, 265]. Immunofluorescent labelling of TREM-2 demonstrated expression in both the murine N9 (Figure 3.1A) and human CHME-5 microglial cell lines (Figure 3.1B). Interestingly, TREM-2 expression was particularly high in the nuclear region in both CHME-5 and N9 microglial cell lines. However, the CHME-5 microglial cell line showed more expression of TREM-2 throughout the cell compared to the N9 cell line (Figure 3.1B). This confirms that TREM-2 expression is expressed in microglial cells and suggests that TREM-2 is located in a peri-nuclear region in these cells.

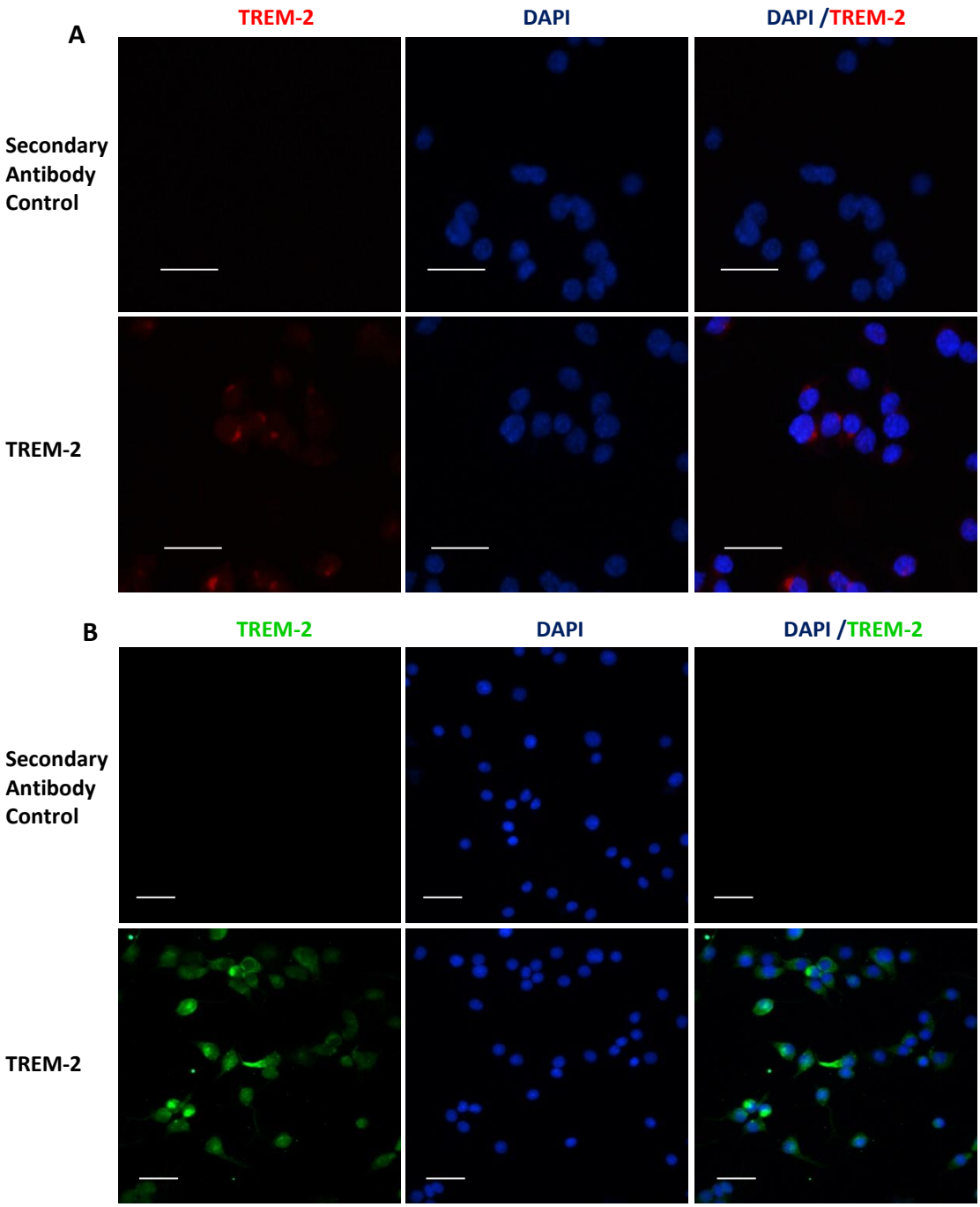

Figure 3.1: TREM-2 protein expression in microglial cell lines by immunofluorescence. TREM-2 protein expression was measured by immunofluorescence in (A) N9 murine microglial cells (RED) and (B) CHME5 human microglial cells (GREEN). DAPI (BLUE) was used to stain the nucleus to help to view localisation of TREM-2 in the cell (n = 3). Scale bar 50 µm.

This interesting pattern of TREM-2 expression led to the use of confocal microscopy to study TREM-2 localisation in N9 cells in more detail. Confocal microscopy analysis showed that although TREM-2 was expressed throughout the cell, the

highest concentration of TREM-2 was localised in dense patches around the nucleus (Figure 3.2). This suggests that in N9 microglial cells, TREM-2 expression is localised to a membrane-bound organelle close to the nucleus and in vesicles or other small membrane bound organelles throughout the cytoplasm.

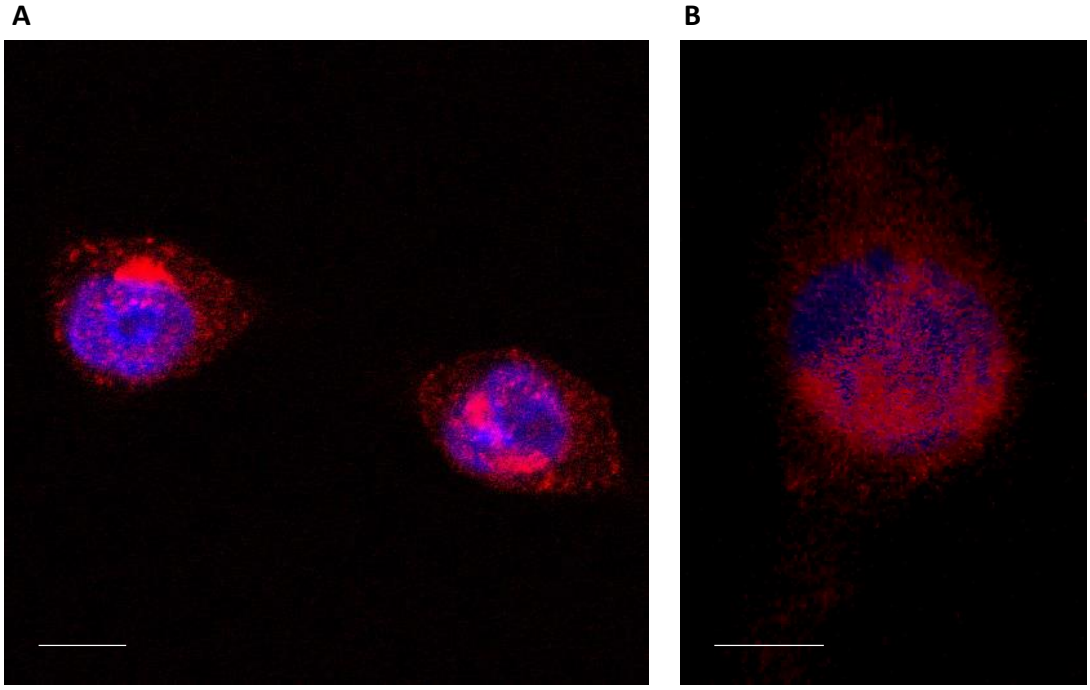

Figure 3.2: Confocal analysis of TREM-2 expression in N9 microglial cells. (A) and (B) Z-stack image showing TREM-2 expression (RED) overlaid with DAPI nuclear staining (BLUE) by immunofluorescence, analysed by confocal microscopy. Scale bar = 10 µm.

To investigate the expression of TREM-2 in myeloid cells further, Figure 3.3 used PMA-differentiated THP-1 cells as a model of macrophages and measured TREM-2 expression throughout the cell. TREM-2 expression and localisation was measured using confocal microscopy. Similarly to the observations in the microglial cell lines, TREM-2 expression was found in a dense circular patch around the nucleus (Figure 3.3). The localisation of TREM-2 around the nucleus in both microglial and macrophage cell lines suggests that TREM-2 may be expressed in membrane bound organelles close to the nucleus.

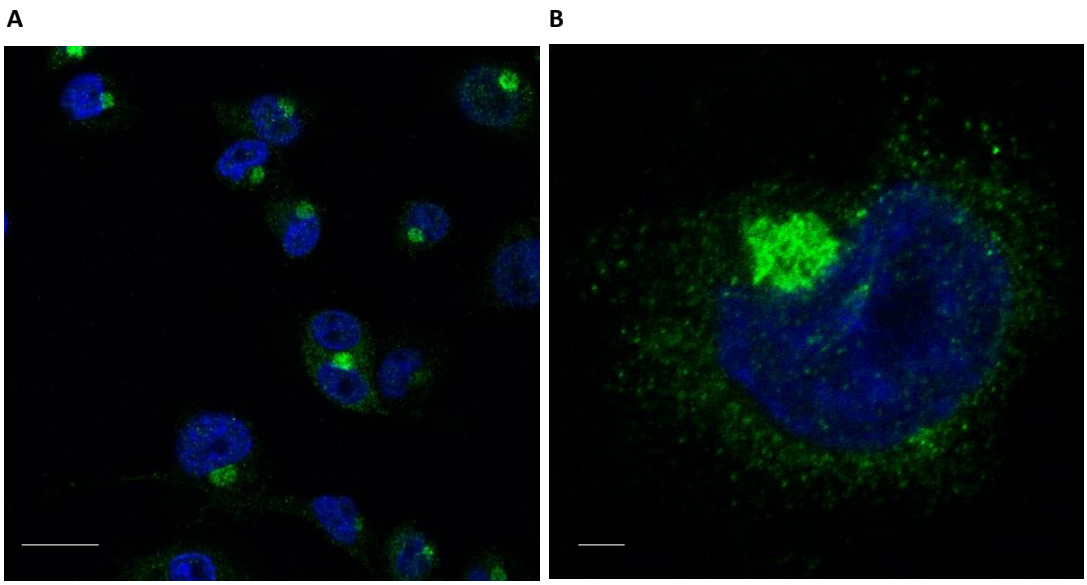

Figure 3.3: Confocal analysis of TREM-2 expression in PMA-differentiated THP-1 cells. (A) and (B) Z-stack image showing TREM-2 expression (GREEN) overlaid with DAPI nuclear staining (BLUE) by immunofluorescence, analysed by confocal microscopy. Scale bars (A) = 20 µm (B) = 2 µm.

To further investigate the high expression of TREM-2 around the nucleus, the absence of TREM-2 in the nucleus was confirmed. Nuclear and cytosolic extracts from N9 cells were compared to total TREM-2 protein expression. As expected, the TREM-2 antibody did not detect TREM-2 expression in the nuclear fraction and very little in the cytoplasmic fraction which may be due to membrane contamination suggesting that TREM-2 is restricted to membrane bound organelles (Figure 3.4).

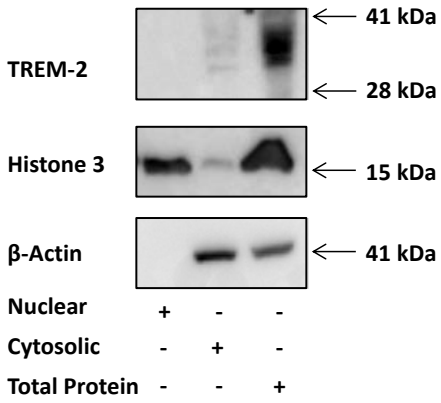

Figure 3.4: TREM-2 protein expression in the cytosol and nucleus of N9 microglial cells. TREM-2 protein expression was measured by western blot analysis for the expression of TREM-2 in the nuclear and cytosolic fractions of N9 cells. Nuclear and cytosolic fractions were compared to total protein. Histone was used as a control for the nuclear fraction and β-actin for the cytosolic fraction (n = 3).

These results suggest that TREM-2 expression is localised around the nucleus in both PMA-differentiated THP-1 macrophages and N9 microglial cells. Two key membrane-bound organelles found close to the nucleus are the endoplasmic reticulum (ER) and the Golgi apparatus. The TREM-2 staining observed in the macrophage and microglial cell lines resembled that of the Golgi apparatus or the centrosome due to its circular shape and location close to the nucleus, therefore a Golgi marker, beta-coatomer-protein (β-COP), was used for dual antibody staining with TREM-2 to analyse TREM-2 expression in the Golgi apparatus. Firstly, TREM-2 and β-COP expression were measured individually to ensure that there was no antibody cross-reactivity in the dual antibody staining experiment (Figure 3.5A and B). As previously shown, TREM-2 expression was observed around the nucleus, with a dense spot of TREM-2 staining on the periphery of the nucleus (Figure 3.5B). This expression pattern was also observed with the β-COP antibody, localised around the nucleus with areas of brighter staining on the periphery of the nucleus (Figure 3.5A).

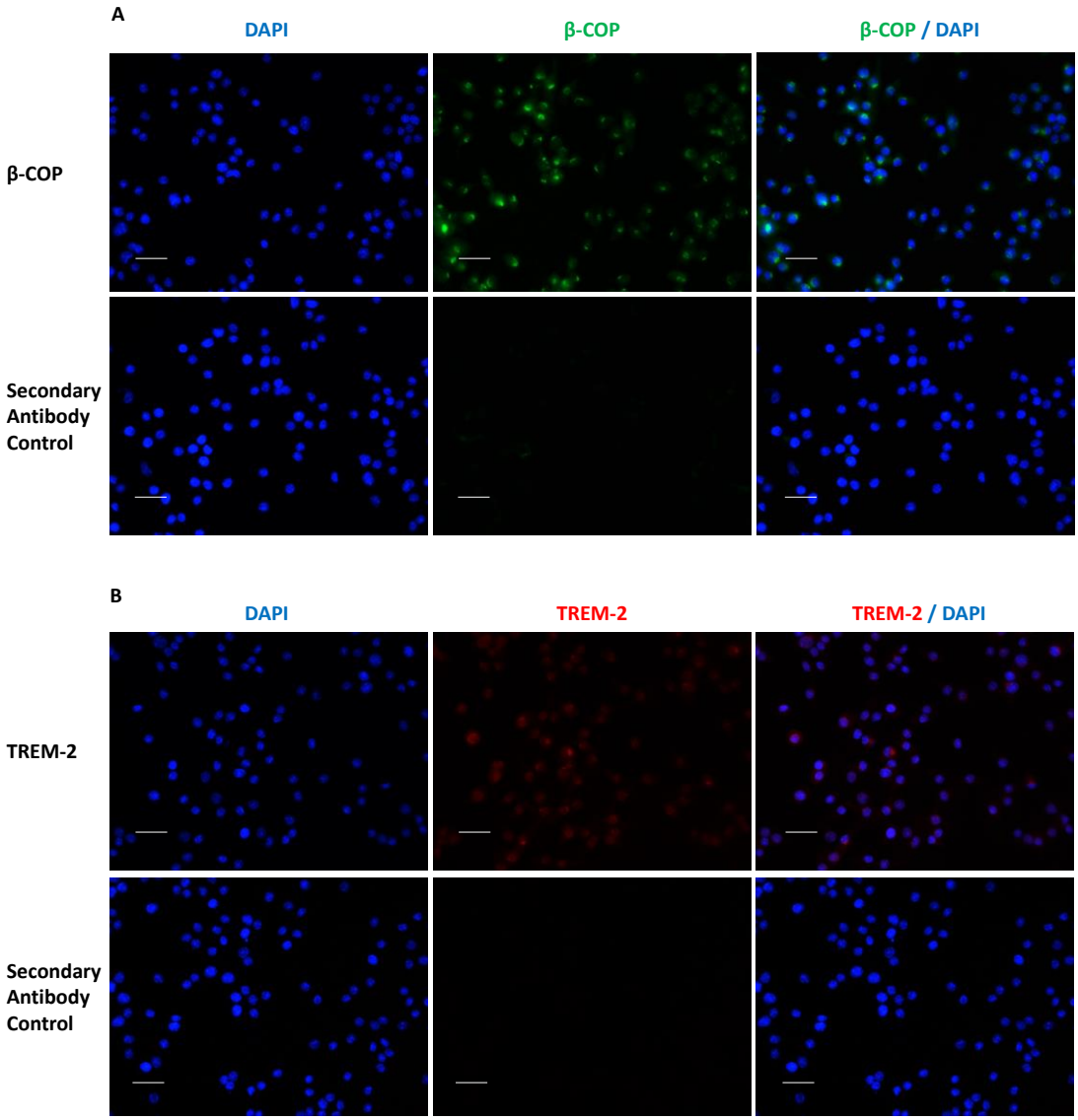

Figure 3.5: TREM-2 and β-COP expression in N9 murine microglia. Expression of (A) TREM-2 (RED) and (B) the Golgi marker β-COP (GREEN) were overlaid with DAPI nuclear stain (BLUE), measured by immunofluorescence in the permeabilized N9 murine microglial cell line (n = 3). 50 µm scale bar.

To observe the co-localisation of TREM-2 and β-COP, immunofluorescence was performed using dual antibody staining for both proteins. The secondary antibody controls showed no non-specific staining after incubation with the secondary antibodies alone (Figure 3.6B). TREM-2 and the Golgi marker β-COP showed some co-localisation in the N9 microglial cells, particularly in the dense spot localised on the periphery of the nucleus (Figure 3.6A). However, TREM-2 expression in some N9 cells was bright throughout the nucleus, a pattern that was not observed with the

β-COP Golgi marker, suggesting that TREM-2 may also be expressed in another organelle near the nucleus such as the ER or centrosome (Figure 3.6A).

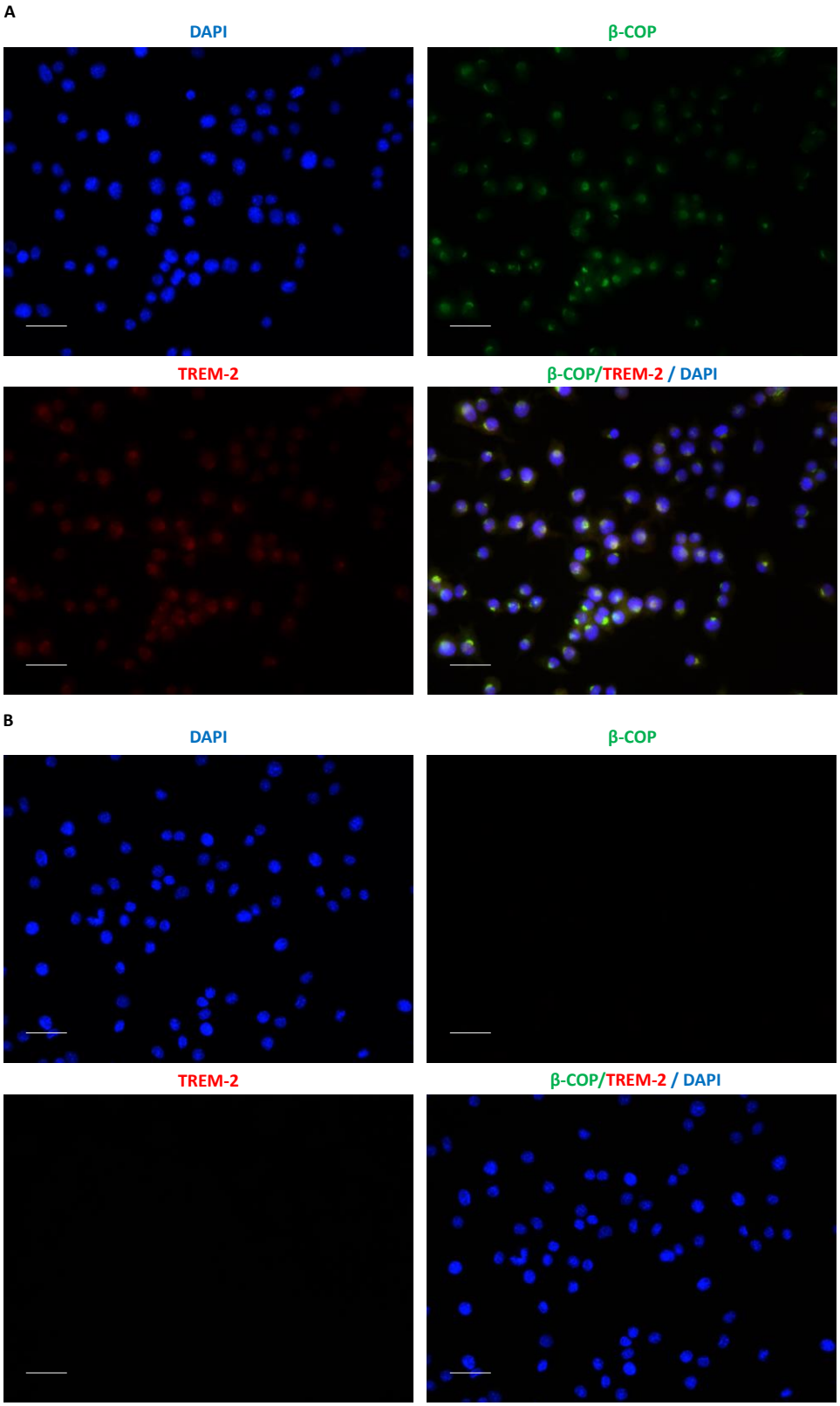

Figure 3.6: Co-localisation of TREM-2 and β-COP expression in N9 murine microglia. (A) Expression of TREM-2 (RED) and the Golgi marker β-COP (GREEN) were overlaid with DAPI

nuclear stain (BLUE), measured by immunofluorescence in permeabilized N9 cells. (B) N9 cells without incubation in primary antibody to control for non-specific binding of the secondary antibody (n = 3). 50 µm scale bar.

Figure 3.7 demonstrates a close-up of the N9 cells in Figure 3.6. The arrows show areas with particularly strong co-localisation of TREM-2 and the Golgi marker β-COP. Together these results confirm the expression of TREM-2 in macrophage and microglial cells and have identified localisation of TREM-2 in the Golgi apparatus in N9 cells.

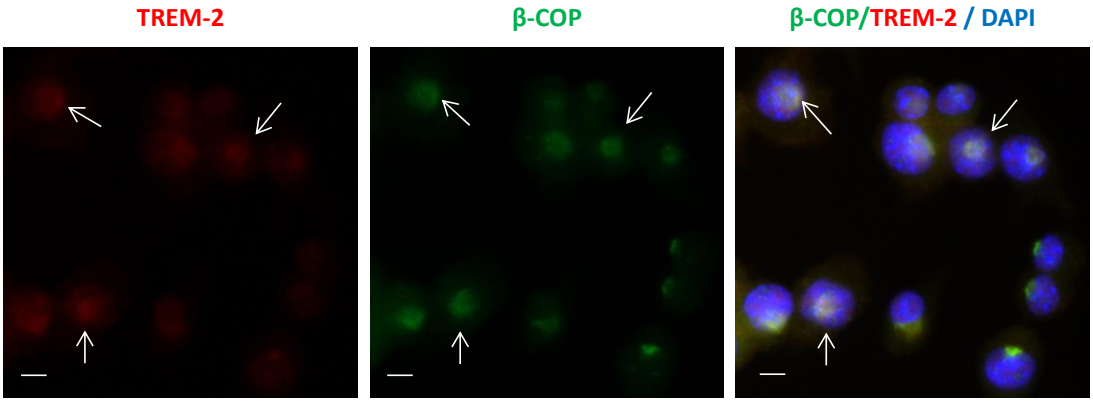

Figure 3.7: Higher magnification of Figure 3.6: Co-localisation of TREM-2 and β-COP expression in N9 microglial cells. Expression of TREM-2 (RED) and the Golgi marker β-COP (GREEN) were overlaid with DAPI nuclear stain (BLUE), measured by immunofluorescence in permeabilized N9 cells. 10 µm scale bar. Arrows highlight good co-localisation of TREM-2 and β-COP.

3.3.2. TREM-2 Expression Screen

In addition to myeloid cells, TREM-2 has also been identified in non-myeloid cell types including genitourinary epithelial cells and liver endothelial cells [241, 257]. After confirming the expression of TREM-2 in myeloid cells by immunofluorescence, myeloid expression of TREM-2 was confirmed by RT-PCR and western blotting, and non-myeloid cells screened for TREM-2 expression. PMA- differentiated THP-1 cells were used as a reference for TREM-2 protein expression and equal concentrations of protein were loaded onto each gel. To compare cells on different western blots,

Figure 3.8 used densitometry analysed by Image J and showed human TREM-2 expression relative to PMA-differentiated THP-1 cells (PMA-THP-1) (Figure 3.8 E and F). Th TREM-2 protein is glycosylated and therefore appears at different weights depending on glycosylation status [278, 298, 359]. The unglycosylated form is believed to be ~28 kDa, as demonstrated in Figure 3.8E. A band was also observed at 32 kDa that is believed to be a glycosylated form of TREM-2 represented in Figure 3.8F. There was also a non-specific band observed at 110 kDa which was confirmed using an IgG control antibody which could be an Fc receptor dimer. TREM-2 protein expression in the mouse cell lines appears around 34 kDa (Figure 3.8D). As expected, myeloid cells including PMA-differentiated THP-1 cells and primary monocyte-derived macrophages expressed high levels of TREM-2 mRNA and protein (Table 3.1 and Figure 3.8A, C and F). High TREM-2 mRNA expression and moderate protein expression was observed in THP-1 monocytes and primary monocytes derived from peripheral blood (Table 3.1). At the protein level, the height and exact weight of the band differed slightly between myeloid cells suggesting that different glycosylated forms of TREM-2 are present (Figure 3.8). The pooled lymphocyte fraction isolated from peripheral blood expressed low levels of TREM-2 mRNA and protein expression.

Table 3.1: TREM-2 mRNA expression in multiple cell types. qRT-PCR analysis of TREM-2 mRNA expression in various primary cells and cell lines. 150 nM cDNA was used for each cell line. mRNA values are expressed as comparative threshold (CT) values for TREM-2 and the control gene (GAPDH) (n = 3).

Cell Type	Average CT GAPDH	Average CT TREM-2
THP-1	16.28	19.97
Primary monocytes	16.92	21.58
PMA-THP-1 cells	18.68	16.08
RAW 264.7	11.38	20.18
HL60	14.42	ND
N9	20.62	19.46
Primary lymphocytes	19.90	32.45
HUVEC	16.93	ND
NCI-H292	13.85	31.84
A549	13.82	ND

16-HBE	14.94	ND
SAEC	13.50	20.97
Caco-2	13.57	ND
M202	12.64	ND
SK-MEL28	13.46	ND
MEL-501	14.58	ND
A2058	14.34	ND
UACC-1273	14.17	ND
3T3-L1	14.54	30.82
MRC-5	13.35	31.96
ARPE-19	20.48	ND
MIO-M1	14.78	ND
SH-SY5Y	13.28	ND

Cells: THP-1, human monocytic cell line; RAW264.7, murine macrophage cell line; PMA-THP-1, PMA differentiated THP-1 cells; HL60, human promyelocytic cell line; N9, murine microglial cell line; primary human lymphocytes and monocytes isolated from PBMCs; HUVECs, human umbilical vascular endothelial cells; NCI-H292 and 16HBE, human airway bronchial epithelial cell lines; A549, human airway type-II alveolar-like epithelial cell line; SAECs, human primary small airway epithelial cells; Caco-2, human epithelial colorectal adenocarcinoma cell line;SK-MEL28, UACC1273, M202, A2058 and MEL-501, human melanoma cell lines; 3T3-L1, murine fibroblast cell line; MRC-5, human fetal lung fibroblast cell line; ARPE19, human retinal epithelial cell line; MIO-M1, human Müller cell line; SH-SY5Y, human neuroblastoma cell line; ND, not detected.

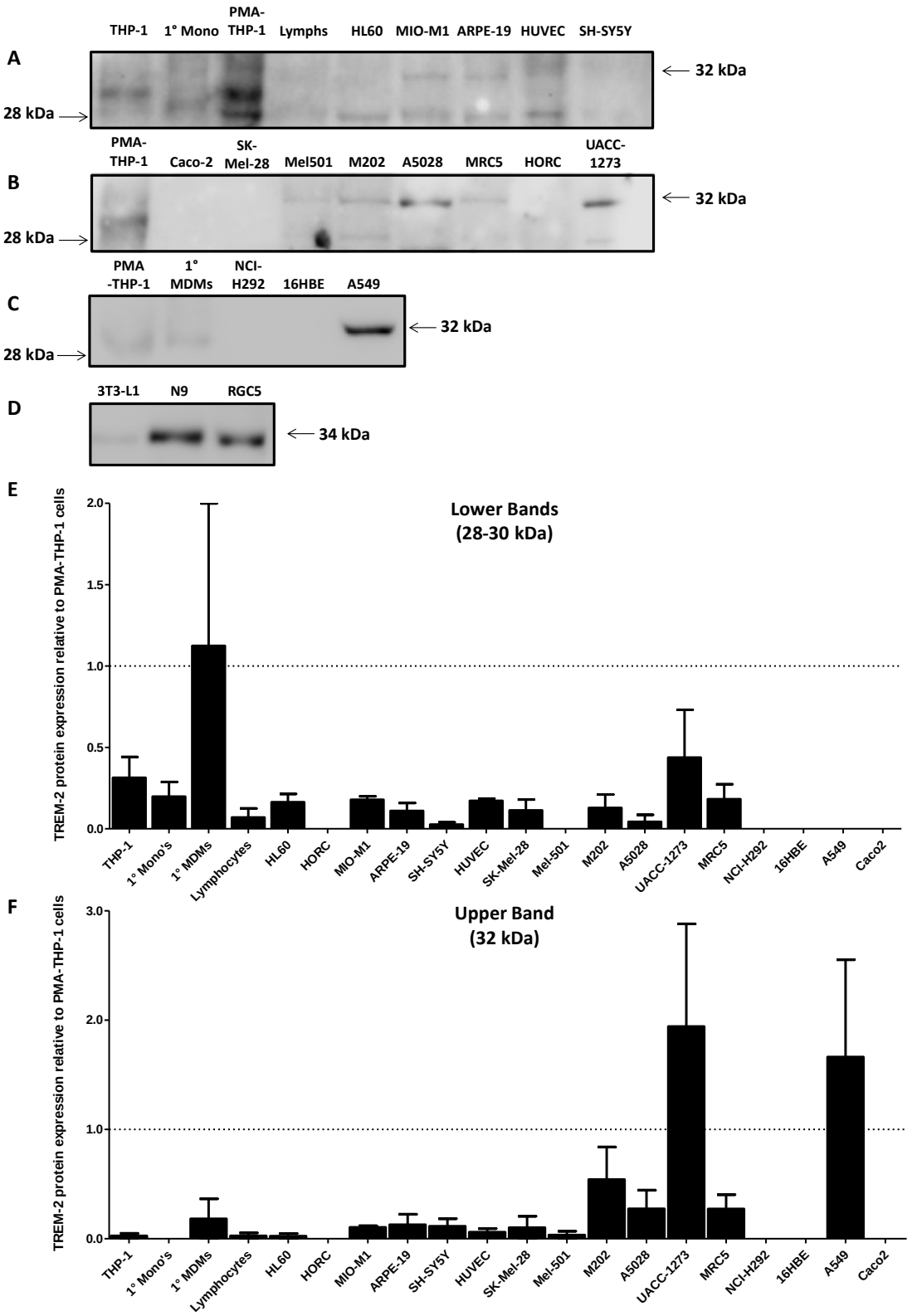

Figure 3.8: TREM-2 protein expression across cell types. (A-D) TREM-2 protein expression was measured by western blot analysis. (A-C) PMA-differentiated THP-1 cells were used as a control for human TREM-2 protein expression represented by the dotted line (……). (D) Mouse TREM-2 protein expression. (E and F) Densitometry values of TREM-2 protein expression, expressed as fold change of the mean densitometry value relative to PMA-differentiated THP-1 cells ± SEM. (E) Represents the band appearing at 28-30 kDa and (F) represents the band

appearing at ~32 kDa. 1° Monos, primary monocytes; 1° MDMs, primary monocyte-derived macrophages; PMA-THP-1, PMA-differentiated THP-1 cells; lymphs, lymphocytes (n = 3).

Since TREM-2 expression has been observed in genitourinary epithelial cells, other epithelial cell types were also investigated for TREM-2 expression. Table 3.1 suggests that the SAECs of the lung express high levels of TREM-2 mRNA (Table 3.1). However, of the airway epithelial cell lines, only the pulmonary bronchial mucoepidermoid carcinoma cell line (NCI-H292) had detectable levels of TREM-2 mRNA (Table 3.1). In contrast, at the protein level, only the human alveolar basal epithelial cell line (A549) seemed to express TREM-2 protein (Figure 3.8 C and F). In this cell line, TREM-2 protein was present at the predicted glycosylated form of TREM-2 (Table 3.1 and Figure 3.8C and F). However, due to the lack of availability of the SAECs, TREM-2 protein expression could not be studied in these cells. TREM-2 mRNA and protein expression were also analysed in a colon epithelial cell line (Caco-2), which showed no detectable levels of TREM-2 mRNA or protein (Figure 3.8B, E and F). This pilot study suggests that TREM-2 may be present in the glycosylated form in some subtypes of airway epithelial cells.

None of the melanoma cell lines had detectable levels of TREM-2 mRNA. However, at the protein level, the A5028 and UACC-1273 cell lines seemed to express high levels of the predicted glycosylated form of TREM-2 (Figure 3.8B and E and F). Western blot analysis suggested that the M202, SK-Mel28 and Mel-501 melanoma cell lines expressed moderate to low levels of TREM-2 in both glycosylated and non-glycosylated forms (Figure 3.8B, E and F). Expression of TREM-2 in the melanoma cell lines were not expected as TREM-2 was not detected at the mRNA level.

The human fibroblast cell line, MRC-5 seemed to express low levels of TREM-2 mRNA (Table 3.1) and moderate levels of TREM-2 protein expression (Figure 3.8B, E and F) suggesting that TREM-2 is expressed in human fibroblasts.

The Müller and retinal pigment epithelial cell lines of the eye (MIO-M1 and ARPE-19 respectively) seemed to express low levels of TREM-2 in both glycosylated and non-glycosylated forms, despite being undetectable at the mRNA level (Table 3.1 and Figure 3.8A, E and F). However, TREM-2 was not detected in the HORC tissue, suggesting that the low levels of TREM-2 predicted in the Müller and pigment epithelial cells of the eye maybe diluted by cells that do not express TREM-2. The

human neuroblastoma cell line SH-SY5Y had no detectable TREM-2 mRNA or protein expression suggesting that TREM-2 is not expressed in these cells.

TREM-2 expression has been previously observed in mouse liver endothelial cells [241]. Figure 3.8E and F suggest that low levels of both glycosylated and non-glycosylated forms of TREM-2 are expressed in HUVECs. However, no TREM-2 mRNA expression was detected by RT-PCR further suggesting that TREM-2 mRNA and protein expression do not always correlate.

Of the few mouse cell lines tested, the N9 microglial and RGC-5 cell lines seemed to express high levels of TREM-2 protein but the 3T3-L1 mouse fibroblast cell line had almost undetectable TREM-2 protein expression (Figure 3.8D-F). The high TREM-2 expression in the murine neuronal precursor cell line, RGC-5 suggests that TREM-2 may be expressed in mouse neuronal precursor cells which contradicts the observations from the SH-SY5Y human neural precursor cell line. In combination with the fact that the mouse fibroblast cell line (3T3-L1) had almost undetectable TREM-2 expression, this suggests that these cell lines are from different origins or that there is a difference in TREM-2 expression in human and mouse neural precursor and fibroblast cells.

Together, this pilot study has shown that non-myeloid cells including melanoma cells, non-myeloid cells of the eye and epithelial cells of the lung may express TREM-2. However, future studies are required to confirm the presence of the glycosylated form of TREM-2 in these cells.

3.3.3. TREM-2 Expression in Human Organotypic Retinal Cultures

Microglia are the main source of TREM-2 expression in the brain. However, microglia are also located in the eye, suggesting that TREM-2 may also function here. There is little evidence regarding TREM-2 expression in the eye. Sun and colleagues demonstrated that TREM-2 is important in corneal inflammation, with increased inflammation and reduced resistance to infection in the absence of TREM-2, but this study did not detail the cell types that express TREM-2 in the eye [368]. Low TREM-2 expression was observed in MIO-M1 and ARPE-19 cell lines by western blot analysis, therefore immunofluorescence was used to analyse TREM-2 expression in primary retinal cultures. In addition, since TREM-2 is increased in cerebral ischaemia, and ischaemia in the eye contributes to the pathogenesis of

ocular conditions including diabetic retinopathy and age-related macular degeneration, the effect of ischaemia on TREM-2 expression in the eye was also studied.

TREM-2 is known to be expressed in microglia and therefore the abundance of microglia in the retina was investigated using the microglial cell marker IBA-1. Figure 3.9 shows the different cell layers of the retina with the nucleus stained blue with DAPI and microglia in green. Microglia are dispersed in the ganglion and Muller/bipolar/horizontal/amacrine cell layers.

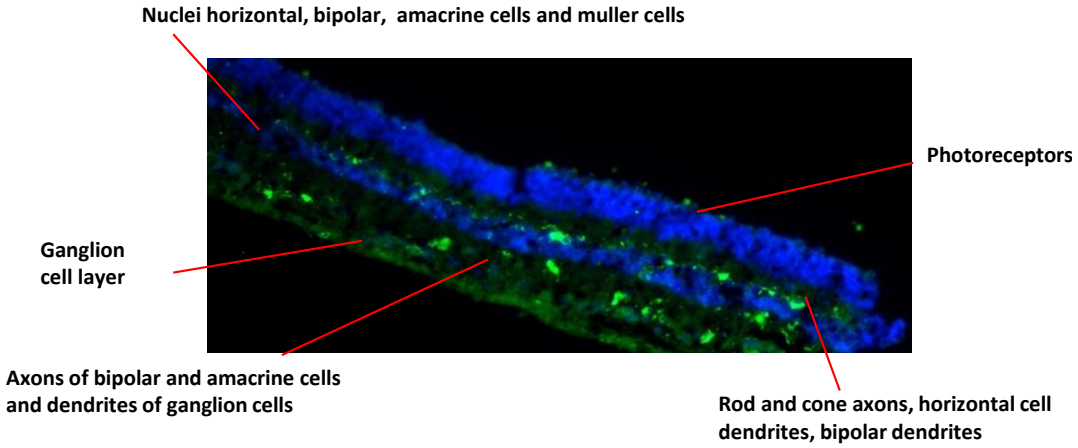

Figure 3.9: Cell types in the HORCs. Immunofluorescence showing the different cell layers of the HORCs. Microglia are highlighted in bright green (IBA-1). DAPI (BLUE) is used to stain the nucleus.

Low TREM-2 mRNA expression (CT value 28.50) was detected in the HORC tissues using qRT-PCR. In addition, 30 min OGD had no significant effect on TREM-2 mRNA expression in this tissue suggesting that TREM-2 is not induced by ischaemia at these time points (Figure 3.10).

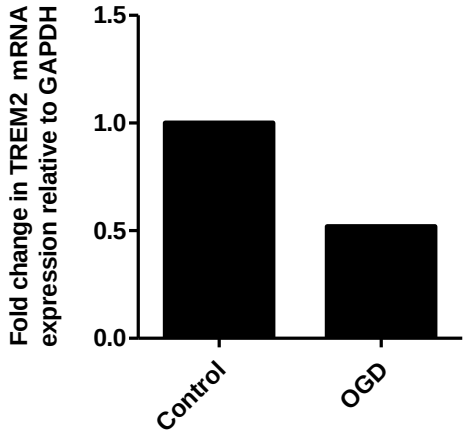

Figure 3.10: TREM-2 expression in HORCs during normal or OGD conditions. HORCs were subjected to OGD or control conditions for 30 min and TREM-2 mRNA expression measured by qRT-PCR. mRNA values are expressed as fold change (relative to the control) normalised to GAPDH (n = 2).

To confirm this effect at the protein level, TREM-2 protein expression was measured in the HORCs using immunofluorescence. TREM-2 expression was low in these tissues and seemed to be expressed in only a few cells (white arrow, Figure 3.11), which are likely to be microglial cells due to the low number present in the tissue. Figure 3.11 also assessed the effect of 3 h ischemia on TREM-2 expression in these samples. Similarly to the mRNA results, ischemia had little to no effect of TREM-2 expression in the HORCs, suggesting that ischemia has no effect on TREM-2 is expression in the eye.

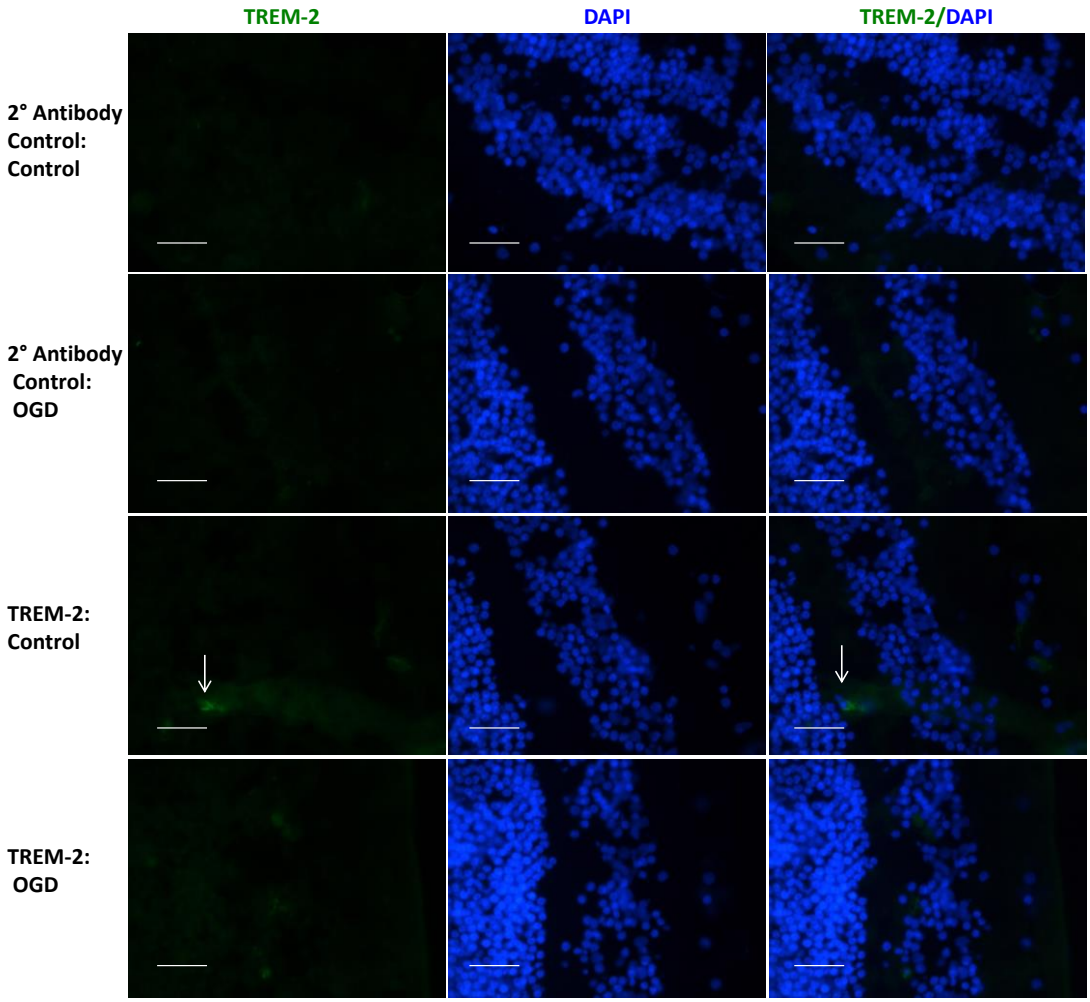

Figure 3.11: TREM-2 expression in the human retina. HORCs subjected to control or OGD conditions for 3 h. TREM-2 expression (GREEN) was analysed by immunofluorescence, DAPI was used to stain the nucleus (BLUE) (n = 3).

3.3.4. TREM-2 Expression in Human Lung Tissue

In addition to the cell lines of the eye, Table 3.1 and Figure 3.8 demonstrate that TREM-2 is expressed in airway epithelium, therefore expression of TREM-2 in human lung tissue was investigated. Paraffin-embedded human lung tissue sections from COPD and asthma patients and healthy controls were assessed for TREM-2 protein expression. The paraffin-embedded post-mortem lung tissue was obtained from Norfolk and Norwich Hospital Pathology Centre. Ethical approval was obtained from the University of East Anglia's Faculty of Health Research Ethics Committee. Of the 14 lungs tested, six were healthy, four were asthmatic, three were COPD and one was both asthmatic and COPD. Table 3.2 shows specific patient details for the

lungs used in this study. Due to the limited availability of lung tissue, there were a variety of causes of death, in particular five of the 14 patients also had pneumonia, which may complicate the results observed in this pilot study (Table 3.2).

Table 3.2: Lung tissue patient information

Patient number	Condition	Cause of death	Age at death	Gender	Other known conditions/factors
1	Healthy	Aspiration of gastric contents	22	Female	-
2	Healthy	Aspiration of gastric contents	35	Female	Non-smoker
3	Healthy	Drug overdose	35	Female	-
4	Healthy	Pneumonia	83	Female	-
5	Healthy	Left ventricular hypertrophy	62	Male	-
6	Healthy	Drug overdose	42	Female	-
7	COPD	Collapsed lung	79	Male	Smoker, bronchopneumonia, malignant mesothelioma.
8	COPD	Drug overdose	52	Male	-
9	COPD	Bronchopneumonia	72	Male	
10	Asthma and COPD	Respiratory failure Bronchopneumonia	74	Male	-
11	Asthma	Meningitis	42	Male	Broncho pneumonia
12	Asthma	Drug overdose	31	Male	-
13	Asthma	Churg-Strauss syndrome	79	Female	-
14	Asthma	Cardiac arrhythmia Myotonic dystrophy	65	Male	-

Figure 3.12 shows immunohistochemical staining of TREM-2 in healthy tissue. Tissue positively stained for TREM-2 is orange/brown. To control for non-specific binding of the primary or secondary antibodies to the tissue, an IgG antibody control was included. The IgG control showed little background staining, whereas incubation with the TREM-2 antibody showed that one out of six of the healthy lungs expressed TREM-2 in what was thought to be the ciliated bronchial epithelium (Patient 5 in Figure 3.12). TREM-2 expression could be found throughout the cell, but seemed to be particularly highly expressed in the cilia. Some ciliated bronchial epithelium expressed TREM-2 along the length of the bronchial lumen (Figure 3.12B), whereas others seemed to express TREM-2 on selected ciliated epithelium and not others (Figure 3.12A). In addition, there were areas that resembled ciliated bronchial epithelium that had no TREM-2 expression, suggesting that TREM-2 is selectively expressed in the ciliated bronchial epithelium of healthy lungs. Unfortunately, the lung sections from four out of six of the healthy patients had no visible ciliated bronchial epithelium and therefore the expression of TREM-2 in ciliated epithelium in these patients could not be studied.

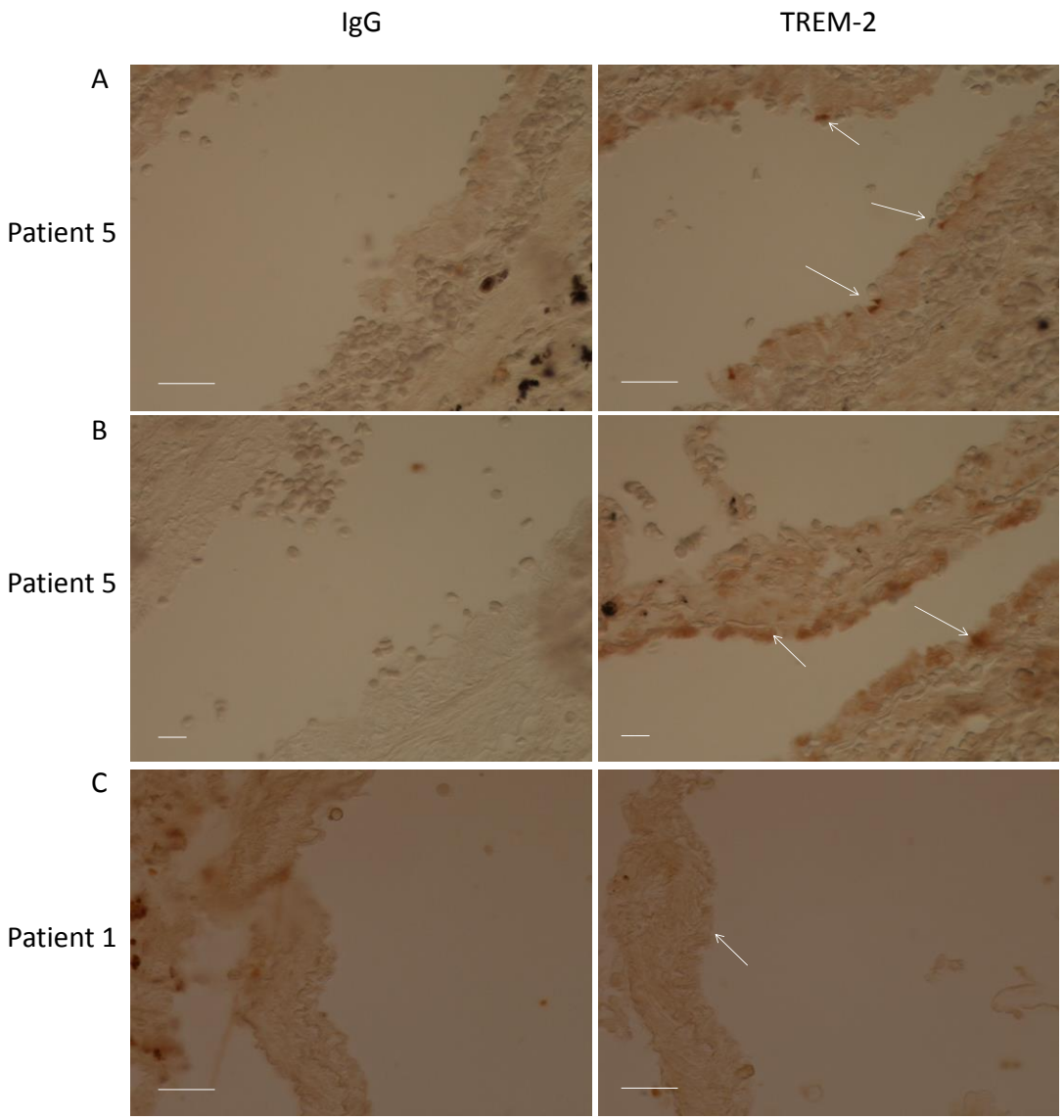

Figure 3.12: TREM-2 is expressed on ciliated bronchial epithelial cells in the healthy human lung. Ciliated epithelium in healthy lung tissue was examined by immunohistochemistry using TREM-2 or IgG control antibodies. Orange/brown staining indicates TREM-2 expression. Healthy lungs from six patients were analysed, ciliated epithelium could only be detected in two out of the six patients. Arrows highlight ciliated epithelium. Scale bar: 30 μm.

Analysis of TREM-2 expression in the lungs of asthmatic patients also suggested that TREM-2 was expressed on ciliated bronchial epithelium in four out of five patients (Figure 3.13). As seen in healthy patients, TREM-2 looked to be expressed most highly on the cilia of these cells (Figure 3.13A). In lungs where ciliated bronchial epithelium could be identified, TREM-2 expression was present (Figure 3.13). However, similarly to the healthy patients, there were some ciliated bronchial epithelial cells that expressed high levels of TREM-2 and others that expressed low

or no TREM-2 suggesting that TREM-2 expression varies depending on the state of the cell.

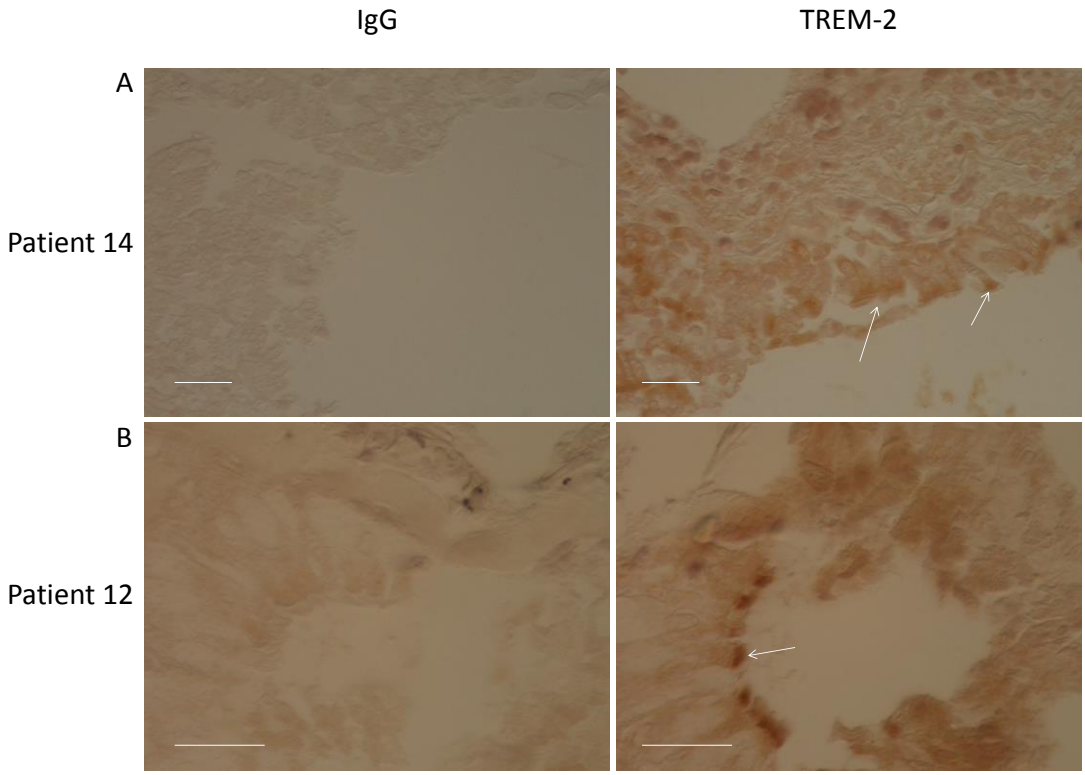

Figure 3.13: TREM-2 is expressed on ciliated bronchial epithelial cells in the asthmatic human lung. Ciliated epithelium in asthmatic lung tissue was examined by immunohistochemistry using TREM-2 or IgG control antibodies. Orange/brown staining indicates TREM-2 expression. Asthmatic lungs from five patients were analysed, ciliated epithelium could be detected in four out of the five patients. Arrows highlight ciliated epithelium. Scale bar: 30 µm.

Koth and colleagues have previously reported increased TREM-2 expression on alveolar macrophages from COPD patients. However, all of the COPD patient samples had denuded epithelium, as shown by Haematoxylin and Eosin staining in Figure 3.14 and therefore TREM-2 expression could not be examined on epithelium of the COPD lungs. COPD patients had many macrophages in the tissue (arrows) that appeared brown due to the high iron content in these cells [(415)]. Macrophages stained for both the IgG control and TREM-2 antibody (arrows), suggesting that this binding to macrophages was non-specific and therefore TREM-2 expression could not be measured in these cells either (data not shown).

Patient 8

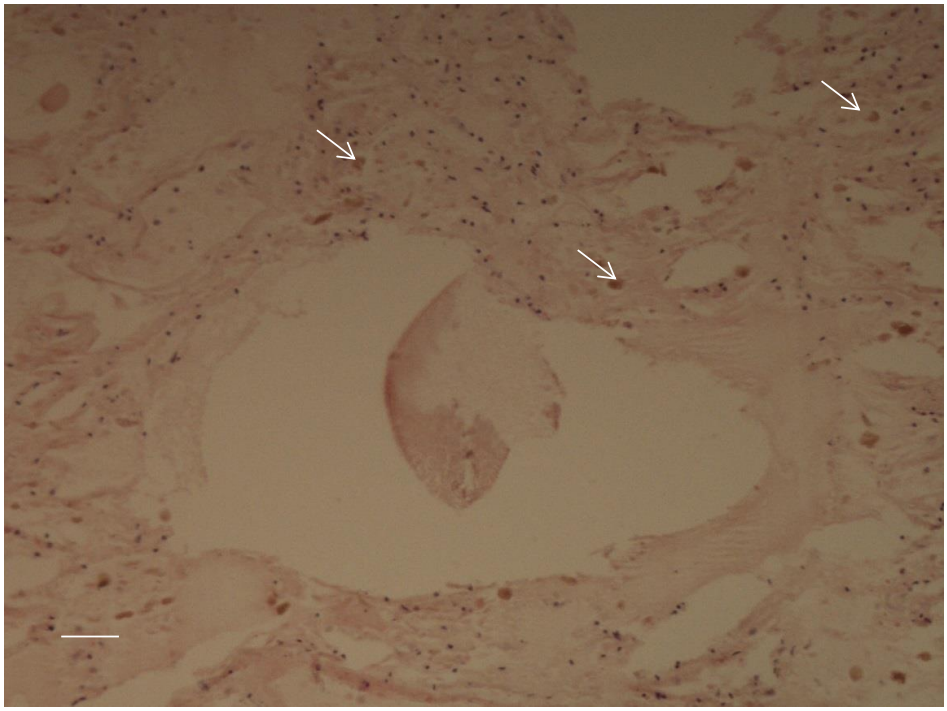

Figure 3.14: Stripped bronchial epithelium in the lung of COPD patients. Haematoxylin and Eosin staining shows a bronchiole where the epithelium has been stripped off. In all three of the COPD cases, no epithelium was identified. Scale bar: 30 µm.

Due to the presence TREM-2 in small airway epithelial cells, TREM-2 expression was also studied in alveolar epithelium. Alveoli were difficult to identify in COPD patients due to denuded epithelium and emphysema-like tissue breakdown and therefore TREM-2 expression could not be assessed in these patients. TREM-2 was not detected on the alveolar epithelium in either asthmatic tissue or normal healthy lung tissue, suggesting that TREM-2 is limited to the ciliated bronchial epithelial cells of the small airways in these patients (Figure 3.15).

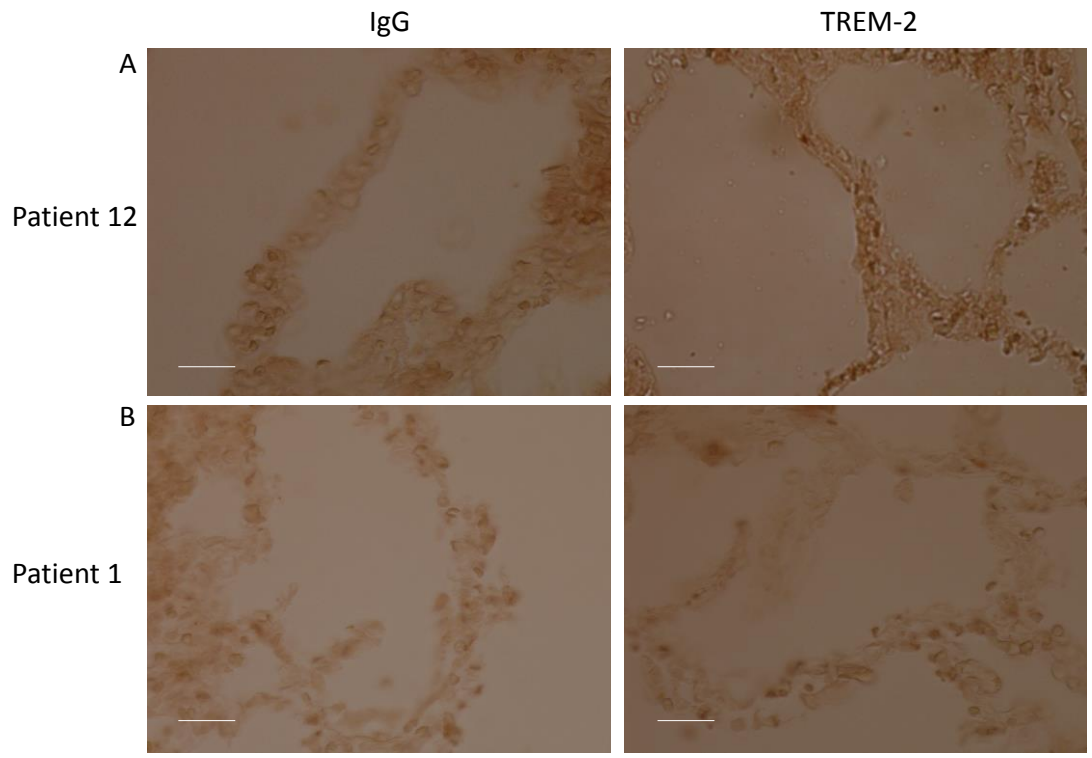

Figure 3.15: TREM-2 is not expressed in alveolar epithelial cells. TREM-2 expression in alveolar epithelium of healthy or asthmatic lung tissue was examined by immunohistochemistry using TREM-2 or IgG control antibodies. Represents five asthmatic lungs and 6 healthy lungs. Scale bar: 30 µm.

In addition to the epithelium, the lung tissue was also analysed for TREM-2 expression in other cells including fibroblasts, smooth muscle cells and endothelial cells. However, TREM-2 was not detected in any lung tissue where the vascular endothelium could be clearly identified (Figure 3.16). In addition, aside from the non-specific staining in the macrophages, TREM-2 expression was not identified in any other areas of the lung. This suggests that TREM-2 expression is not found in smooth muscle cells, endothelial cells or fibroblasts in the human lung.

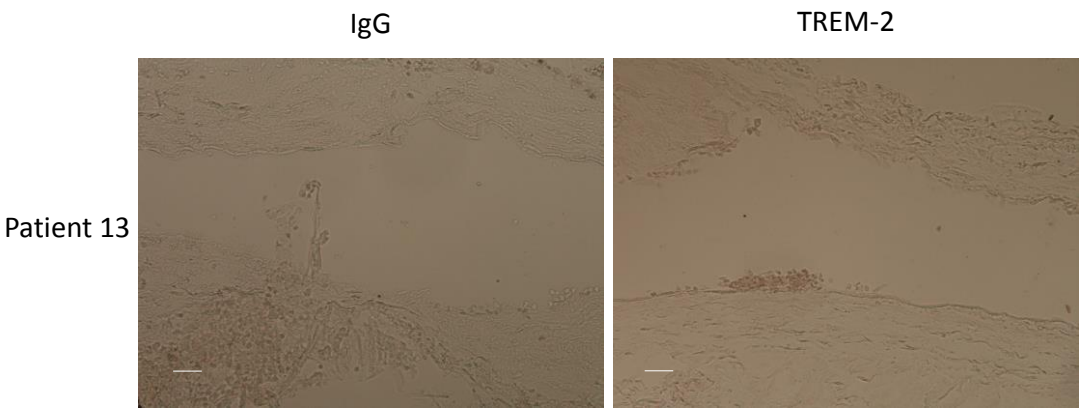

Figure 3.16: TREM-2 is not expressed on lung endothelial cells. TREM-2 expression was measured in the vascular endothelium in asthmatic lung tissue by immunohistochemistry using TREM-2 or IgG control antibodies. Scale bar: 30 µm.

The table below summarises the findings from this study detailing bronchial epithelial denudation and TREM-2 expression from the tissues of each patient (Table 3.3). This study shows that of the two healthy lungs where ciliated epithelium could be identified, TREM-2 expression was identified in one. In asthmatic patients, 4 out of 5 lungs had ciliated bronchial epithelium and all of those expressed TREM-2. Finally, the bronchial epithelium of COPD patients could not be identified due to denudation and therefore TREM-2 expression could not be studied in these patients. Together, these results indicate that TREM-2 is expressed on the cilia of bronchial epithelial cells in healthy and asthmatic lungs.

Table 3.3: TREM-2 expression in healthy, asthma and COPD lung tissue. Overview of TREM-2 expression and presence or absence of bronchial epithelium in the lung tissue samples from each patient.

Patient Number	Disease	Bronchial ciliated epithelium present?	TREM-2 expression
1	Healthy	Yes	No
2	Healthy	None	No
3	Healthy	None	No
4	Healthy	None	No
5	Healthy	Yes	Yes
6	Healthy	None	No
7	COPD	None	No
8	COPD	None	No
9	COPD	None	No
10	Asthma	Yes	Yes
11	Asthma and COPD	Yes	Yes
12	Asthma	Yes	Yes
13	Asthma	None	NA
14	Asthma	Yes	Yes

3.4. Discussion

TREM-2 expression is mostly reported in myeloid cells, but has also been identified in non-myeloid cells including some epithelial cell types [81, 240, 241, 257]. This study found that TREM-2 was located in the Golgi apparatus in microglia with similar staining observed in the macrophages. As expected, TREM-2 mRNA and protein expression were high in the myeloid cell lines. However, the pilot study suggested that TREM-2 may also be expressed in non-myeloid cells, including cell lines and primary epithelial cells of the lower respiratory tract and for the first time on cilia of bronchial epithelial cells in healthy and asthmatic lung tissue. The melanoma cell lines also seemed to express TREM-2, suggesting that TREM-2 may function in melanoma. TREM-2 expression in the retina was low apart from a few positive cells in the primary retinal cultures (HORCs) which are predicted to be microglia. Altogether, this study shows that TREM-2 expression is not restricted to myeloid cells and that it may be expressed in melanoma and airway epithelial cells.

This study confirmed TREM-2 expression in microglia and macrophage cell lines by immunofluorescence before studying the localisation of TREM-2 expression. Most studies to date have focused on the expression of TREM-2 on the cell surface. However, this study and others have shown that TREM-2 is also expressed intracellularly [259, 264]. Immunofluorescence showed TREM-2 expression in PMA-differentiated THP-1 cells (model of macrophages) and murine N9 microglial cells, showing a high expression of TREM-2 intracellularly around/over the nucleus. Since TREM-2 can also act as a soluble receptor, this study used fractionation and confirmed that TREM-2 was not in the nucleus but showed some co-localisation with the Golgi marker β-COP in the microglial cell line, suggesting that TREM-2 is expressed in the Golgi apparatus. Confocal microscopy of TREM-2 expression in the macrophage cell line also suggests that TREM-2 is expressed in the Golgi apparatus, as the localisation is similar to that in N9 cells. In addition, there was also expression of TREM-2 in other locations around the nucleus other than the Golgi apparatus in the microglial cell line, suggesting that TREM-2 is also expressed in other organelles around the nucleus including the ER and centrosome. This centrosomal-looking staining was most apparent in the PMA differentiated THP-1 cell line. This is particularly interesting since TREM-2 has been shown to alter cell shape and morphology through actin dynamics and therefore may also play a role in microtubule dynamics. To test this theory, future studies will use centrosome markers such as ninein and use confocal microscopy to investigate the expression

of TREM-2 in the centrosome in PMA-differentiated THP-1 cells and primary macrophages. Whether TREM-2 plays a role in the Golgi apparatus or is merely stored there is not yet known. However, the Golgi apparatus is important in phagocytosis, a process where TREM-2 plays an important role [81, 416]. The Golgi apparatus is required for the trafficking of proteins required for efficient phagocytosis from the ER and Golgi apparatus via endosomes and lysosomes to the phagosome [416-418]. The expression of TREM-2 in the Golgi apparatus may be, in part, how the Golgi apparatus regulates phagocytosis, by regulating phagocytic proteins such as TREM-2 at the phagocytic cup. TREM-2 may also regulate the other proteins in the phagocytic cup through its location in the Golgi apparatus. Future work aims to investigate the role of TREM-2 and the Golgi apparatus in the formation and composition of the phagocytic cup and how this effects phagocytosis.

This study also showed high levels of TREM-2 expression in the macrophage and microglia cell lines by western blot analysis and qRT-PCR, which supports the immunofluorescence data and the observations in the literature [240, 243]. However, not all myeloid cells have been shown to express TREM-2. Turnbull and colleagues reported that peripheral blood monocytes do not express TREM-2 [240]. In contrast, this study showed that primary monocytes and the monocyte THP-1 cell line expressed TREM-2 protein and contained comparable TREM-2 mRNA expression to microglia and macrophages. The differences observed in this study compared to Turnbull and colleagues are likely to be due to the different methods of protein detection, as Turnbull and colleagues analysed cell surface TREM-2expression, whereas this study analysed total TREM-2 protein in the cell and since TREM-2 expression is high in the Golgi apparatus of myeloid cells, this may be the main source of TREM-2 in monocytes. Interestingly, the pre-myelocytic HL60 cell line contained no detectable TREM-2 mRNA expression and low protein expression, suggesting that TREM-2 expression increases with differentiation and that there may be a mechanism in monocytes that prevents TREM-2 mRNA translation that can be switched off with differentiation. In agreement with our results, other groups have observed increased TREM-2 protein expression after differentiation of peripheral monocytes into osteoclasts, dendritic cells and macrophages [299, 309]. This increase in TREM-2 expression is thought to be important for differentiation into macrophages and dendritic cells, suggesting that TREM-2 plays an important role in differentiation [281]. The lymphocyte fraction of PBMCs was also assessed for TREM-2 expression, which was only just detectable at both the protein and mRNA level. Piccio and colleagues showed no TREM-2 expression in CD14 negative cells

(predominantly lymphocytes), suggesting that the low TREM-2 expression may be due to low level contamination with myeloid cells [278].

Fibroblasts are another key cell type important for the regulation of tissue homeostasis and the regulation of inflammation [419-421]. MRC-5 cells seemed to express moderate levels of both the glycosylated and non-glycosylated forms of TREM-2 protein. TREM-2 expression has also been observed in fibroblasts from rheumatoid arthritis joints and at the mRNA level in the 3T3-L1 cell line, further suggesting that TREM-2 is expressed in some fibroblast cell types [244, 374]. To understand this further, future studies will analyse TREM-2 expression in primary fibroblasts in cell culture or in human or mouse tissue identifying the fibroblasts using a cell marker for example fibroblast-specific protein 1 [422].

TREM-2 expression was also investigated in melanoma cell lines, including UACC-1273, SK-Mel-28, Mel-501, M202 and A2058. Although TREM-2 mRNA was not detected in any of the melanoma cell lines tested, the protein data suggested that the predicted glycosylated form of TREM-2 was highly expressed in the UACC-1273 and A5028 cell lines and moderately expressed in M202, A2058, SK-Mel-28 and Mel-501 cell lines. In addition, the non-glycosylated form of TREM-2 was also expressed in these cells but at lower levels. This mismatch between TREM-2 protein and mRNA expression could be for four reasons: (i) low TREM-2 turnover [423], (ii) mutations in the TREM-2 gene in these cell lines preventing primer binding, (iii) TREM-2 primers bind only transcripts 1 and 1x and not transcript 2 and therefore the cells that express TREM-2 protein but not mRNA may only express TREM-2 mRNA transcript 2, (iv) non-specific binding of the TREM-2 antibody at the predicted glycosylated weight. Future work will use TREM-2 siRNA to confirm that the band at the higher weight is in fact TREM-2. Following this, these cells could be sequenced for mutations in the TREM-2 gene. Despite these limitations, the protein data from this study suggests that melanoma cells may express high levels of TREM-2 suggesting that TREM-2 may play be important in melanoma. Since TREM-2 has not been previously studied in cancer, this research suggests that this may be an interesting area to pursue in the future.

TREM-2 has also been identified in endothelial cells of the liver and in the microvasculature around arthritic joints [241, 244]. However, this study found no detectable TREM-2 mRNA in HUVECs and only low levels of protein. In addition, TREM-2 was not expressed on endothelial cells of the vasculature in the lung as

identified by immunohistochemistry suggesting that TREM-2 is only expressed on specific types of endothelial cells.

TREM-2 expression has been previously identified in genitourinary epithelial cells, which led us to investigate TREM-2 expression in other epithelial cell types [257]. TREM-2 expression has been identified in the gastrointestinal tract (GIT), but its expression in epithelium has not been measured [337]. TREM-2 mRNA was not detectable in the colon epithelial cell line, Caco2. This was also the case at the protein level, suggesting that TREM-2 is not expressed in colon epithelium. However, future work is required to confirm this finding in human or mouse primary cells, as this cell line may not be a true representative of the colon epithelium. It would also be interesting to measure TREM-2 expression in other parts of the GIT epithelium as well as in different cell types including paneth cells, goblet cells and entero-endocrine cells, as these carry out very different functions and therefore may vary in TREM-2 expression.

The lung epithelium is becoming increasingly recognised for its similarities to the GIT epithelium [424]. However, despite the role of these cell barriers in immune functions, very few studies have investigated TREM-2 expression in these cell types. Rigo and colleagues reported TREM-2 expression in normal bronchial epithelial (NBE) cells in *in vitro* culture [425]. A similar finding was also observed by Sun and colleagues at the mRNA level in a human bronchial epithelial cell line (HBEC), but aside from this, there is very little information regarding cellular expression of TREM-2 in the lung [374]. This study showed that human SAECs express TREM-2 mRNA. The A549 type II alveolar-like epithelial cell line had no detectable TREM-2 mRNA but Western blot analysis suggested that the predicted glycosylated form TREM-2 was highly expressed in this cell line but the NCI-H292 mucoepidermoid carcinoma and 16-HBE bronchial epithelial cells lines had no TREM-2 protein expression. This varied expression of TREM-2 in these cells suggests that either TREM-2 expression is altered in lung cancer or that TREM-2 expression varies throughout the lung and therefore varies between types of epithelial cells. It is unusual to see that the A549 cells have no detectable TREM-2 mRNA but such high expression of TREM-2 protein. However, Sun and colleagues showed that the A549 cell line has low levels of TREM-2 mRNA and therefore it may be that these levels are below the detection limit of this system but are adequate for TREM-2 protein expression [374]. No detectable mRNA but moderate protein expression was also observed in the melanoma cell lines and therefore this may also be due to a number of other reasons discussed above. Again, similarly to

the melanoma cell lines, future work on this study will use TREM-2 KD to confirm TREM-2 protein expression and gene sequencing will be performed to identify any mutations.

TREM-2 is increased in the lung of COPD patients and in the house dust mite model of allergy, but has only been measured in macrophages [242, 367]. To further validate TREM-2 protein expression in the human lung, TREM-2 expression was measured using immunohistochemistry staining of human lung tissue from healthy, COPD and asthmatic patients. This study suggests that TREM-2 expression was located on the bronchial epithelium of the small airways, which supports the mRNA data showing that the SAECs express TREM-2, and published data showing that TREM-2 is expressed in the HBEC and NBE cells [374, 425]. In all cases, TREM-2 expression seemed to be particularly high on structures resembling the cilia of bronchial epithelial cells. A similar observation was observed on genitourinary epithelial cells by Quan and colleagues, suggesting that TREM-2 may have a role in ciliary functions [257]. Cilia are hair-like projections on the luminal surface of bronchial epithelial cells. These structures increase the surface area for gas exchange in the lung, and clear mucus, unwanted debris and pathogens from the lung [426]. Cilia beat frequency can be enhanced by TLR ligands: LPS, CpG, flagellin and Pam3CSK4, to mediate pathogen removal from the lungs [426]. TREM-2 is also important for pathogen clearance by binding to PAMPs on the surface of bacteria and increasing NO production, two mediators that enhance cilia beat frequency [261, 288, 426]. In addition, cilia beating is regulated by intracellular calcium oscillations and since TREM-2 activation increases intracellular calcium, this may be another reason for the location of TREM-2 in the cilia [259, 427].

Despite the high TREM-2 expression in the A549 alveolar cell line, TREM-2 expression was not found on the alveolar epithelium of primary human lung tissue in any of the patients tested. This suggests that TREM-2 expression in the A549 adenocarcinomic cell line is due to the cancerous nature of the cells. This also suggests that TREM-2 may be increased in lung cancers, which is an area that has not been previously studied and therefore would be an area of interest. Unfortunately, epithelium could not be detected in any of the three COPD patient lungs and therefore TREM-2 expression in the epithelium of COPD patients could not be assessed. Denudation of the airway epithelium is a common occurrence in both asthma and COPD and therefore the absence of epithelium could have been predicted [428]. In addition, emphysema, a common pathology of COPD, causes alveolar destruction making it very difficult to identify any alveoli in these patients. It

is also likely that the tissue processing also caused epithelial denudation, since the healthy controls also had denuded epithelium, therefore, future studies will optimise the protocol to reduce epithelial shedding during the procedure. In addition, TREM-2 expression was not found in any other location in the lung suggesting that neither smooth muscle cells, fibroblasts nor endothelium express TREM-2 in the lung. Several studies have reported TREM-2 expression in the brain [259, 274, 429]. However, the expression of TREM-2 in the brain is high in microglial cells although low expression has been observed in hippocampal neurons [81, 149, 258, 259]. In agreement with this, this study found that TREM-2 was not expressed in the human SH-SY5Y neuroblastoma cell line. The eye is heavily innervated by the CNS and contains unique cells similar to those found in the brain including photoreceptors, ganglion cells and Müller cells [430]. TREM-2 has been previously been shown to be increased in the eye after infection [368]. However, very little is known about cell types in the eye that express TREM-2. This study found that there was no detectable TREM-2 mRNA in the MIO-M1 and ARPE-19 cells, and TREM-2 mRNA expression in the HORCs was low but detectable. MIO-M1 and ARPE-19 cells seemed to express low levels of TREM-2 protein comparable to that of HL60 and THP-1 cells. Observing TREM-2 protein expression by immunofluorescence, the HORCs contained little TREM-2 expression matching the western blot data. However, there were some TREM-2 expressing cells present in the HORC tissue which are likely to be microglia. There are many eye diseases associated with dysfunction of the retina, many of which are caused by or lead to ischaemia e.g. age-related macular degeneration [431]. Ischaemia had no effect on TREM-2 expression at the time points observed. However, TREM-2 expression may be increased over longer time points, over which fresh HORC tissue is difficult to maintain. In addition, it may be that *in vivo*, TREM-2 is increased during ischaemia due to an influx of cells including microglia or macrophages which cannot be seen in this model.

Of the mouse cell lines tested, this study suggested that TREM-2 was highly expressed in the N9 microglial and RGC-5 neural precursor cell lines, and almost undetectable in the 3T3-L1 fibroblast cell line. This low expression in the fibroblast cell line may be in part due to the high expression of TREM-2 in the other cell lines, reducing the ability to detect the 3T3-L1 band using the G:BOX Imager and therefore it is difficult to directly compare the 3T3-L1 cell line to the human fibroblast cell line. The high expression of TREM-2 in the RGC-5 cell line was unexpected, since the human neuroblastoma cell line expressed no TREM-2. However, the RGC-5 cells were originally thought to be rat retinal ganglion cells, but Van Bergen

and colleagues later identified them as mouse neuronal precursor cells, therefore since this has not been tested in this lab, the exact origin of these cells cannot be confirmed and therefore must be treated with caution and may be the reason for the species differences observed here [393].

This study has shown TREM-2 expression in both glycosylated and non-glycosylated forms. Chapter 5 shows that the non-glycosylated form can be knocked out with TREM-2 siRNA in THP-1 cells. However, THP-1 cells do not express the 32 kDa form and therefore this form has not been knocked down. Despite the evidence in the literature for glycosylated and non-glycosylated forms of TREM-2 [278, 298, 359] and TREM-2 validation around this weight [432], the expression of TREM-2 at 32 kDa should be validated in future studies using siRNA KD to confirm this band as TREM-2. In addition, mouse TREM-2 was identified at the higher molecular weight of ~34 kDa, this weight is supported by previous studies who have identified mouse TREM-2 around this weight [259].

3.5. Summary

In summary, this study has shown that TREM-2 expression is not limited to myeloid cells. TREM-2 expression was found in a number of cell types but, of particular interest, in melanoma and lung cells. TREM-2 in the lung was found in ciliated bronchial epithelial cells with high levels located on the cilia, suggesting that TREM-2 may be required for cilia function in the lung. In microglial and macrophage cell lines, TREM-2 expression was localised to the Golgi apparatus, which has an important role in phagocytosis. In addition, the identification of TREM-2 in non-myeloid cell types including melanoma and lung cells reveals the potential to discover more functions of TREM-2 in these cell types in health and disease.

4. Regulation of TREM-2 Expression by Inflammatory Mediators in Myeloid Cells

4.1. Introduction

Monocytes circulate in the blood stream until a chemokine gradient attracts them to a site of injury or inflammation [23]. In response to pro-inflammatory stimuli, monocytes become activated and release numerous pro-inflammatory mediators that contribute to the response to infection or to the disease process [433]. They also migrate into inflamed tissues and differentiate into macrophages and dendritic cells, depending on the surrounding environment. However, monocytes can also function in tissues in their undifferentiated forms e.g. in animal models of obesity and atherosclerosis [434, 435] and in the lung [436] and therefore contribute to inflammation in tissues as well as in the blood.

Some of the most potent activators of monocytes are TLR ligands [28]. These include LPS, peptidoglycan and Pam3Cys, which activate TLR receptors and the NFκB pathway, resulting in the secretion of a multitude of inflammatory mediators including TNF-α, IL-1β, IL-6, IL-8 and anti-inflammatory mediators such as IL-10 [28, 437-439]. These inflammatory mediators are also present during inflammation in disease and following injury [440-442]. These pro-inflammatory mediators cause influx of many other cell types to the site of injury including T-cells and neutrophils, increasing inflammation and tissue damage [228].

Immune cells also secrete anti-inflammatory cytokines including IL-10, IL-4 and IL-13. These cytokines can reduce the secretion of pro-inflammatory mediators including IL-1β and TNF-α, and drive macrophages into a more anti-inflammatory M2-type phenotype, promoting the resolution of inflammation [443, 444]. The process of resolution of inflammation is very important to prevent chronic inflammation, which is a major cause and consequence of many inflammatory diseases.

TREM-2 expression is increased in many inflammatory conditions, including COPD, MS and pathogen infections [242, 243, 366]. As shown in Chapter 3 Section 3.3.2, basal TREM-2 protein expression in monocytes is low and is increased upon differentiation into macrophages [240, 281, 302]. However, monocytes expressing high levels of TREM-2 have been identified in the CSF of MS patients, demonstrating that TREM-2 is increased on monocytes in inflammatory conditions [278]. Despite this increase in TREM-2 expression in inflammatory conditions, pro-inflammatory mediators including LPS and IL-1β have been shown reduce TREM-2 expression in macrophages [240, 241], while mediators responsible for increasing TREM-2 expression are currently unclear. The potential role of TREM-2 in the resolution of inflammation and the reduction in TREM-2 expression by pro-inflammatory

mediators suggest that anti-inflammatory mediators may cause the increase in TREM-2 expression observed in disease.

4.2. Aims

The aims of this study were to:

- Analyse the effect of various pro- and anti-inflammatory mediators on TREM-2 expression in primary human monocytes and monocytic and macrophage cell lines
- Investigate the signalling pathways required for TREM-2 regulation in these cells.

4.3. Results

4.3.1. TREM-2 Regulation by TNF-α and TLR ligands

To understand the regulation of TREM-2 expression in inflammatory conditions, the effect of various pro-inflammatory mediators on TREM-2 expression in RAW 264.7 murine macrophages and THP-1 monocytes were studied. RAW 264.7 macrophages are a well-characterised model of murine blood monocyte-derived macrophages and THP-1 cells have previously been characterised in our laboratory as a good model of LPS and TNF-α signalling pathways in monocytes [445, 446].

Figure 4.1 demonstrates that LPS downregulates TREM-2 mRNA expression in RAW 264.7 murine macrophages ($p \leq 0.05$), demonstrating the ability of this pro-inflammatory mediator to reduce TREM-2 expression in macrophages.

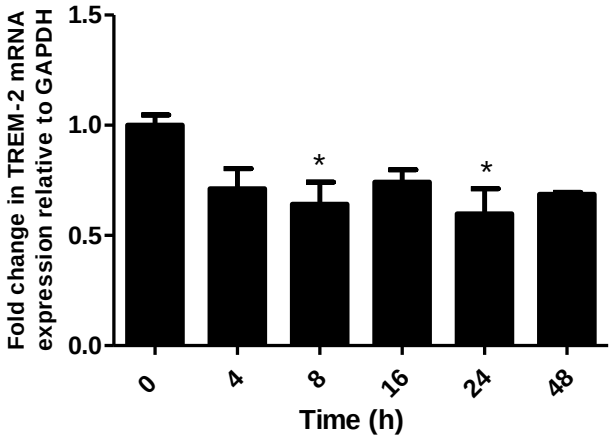

Figure 4.1: LPS reduces TREM-2 mRNA expression in RAW 264.7 macrophages. RAW 264.7 murine macrophages were incubated with 10 µg/mL LPS (TLR4) for 4-48 h. TREM-2 mRNA was measured by qRT-PCR. mRNA values are expressed as fold change (relative to the control) normalised to GAPDH ± SEM (n = 3). *$p \leq 0.05$ (one-way ANOVA with post hoc Dunnett's test).

In THP-1 monocytes, LPS significantly increased TREM-2 mRNA expression after 8 h of stimulation ($p \leq 0.05$) but significantly reduced TREM-2 expression ($p \leq 0.05$) after 24 h (Figure 4.2A). The TLR2 agonists Pam3Cys and peptidoglycan regulated TREM-2 mRNA expression with similar kinetics, showing an increase at 8-16 h and a reduction at 24 h (Figure 4.2C and D). TREM-2 mRNA expression was

moderately reduced at 4 h, to varying degrees between TLR agonists (Figure 4.2). TNF-α treatment resulted in a non-significant reduction in TREM-2 expression by 4 h ($p = 0.077$), similar to the TLR agonists (Figure 4.2B). However, in contrast, the reduction remained for at least 24 h following TNF stimulation. These results support the current evidence showing that pro-inflammatory mediators reduce TREM-2 mRNA expression.

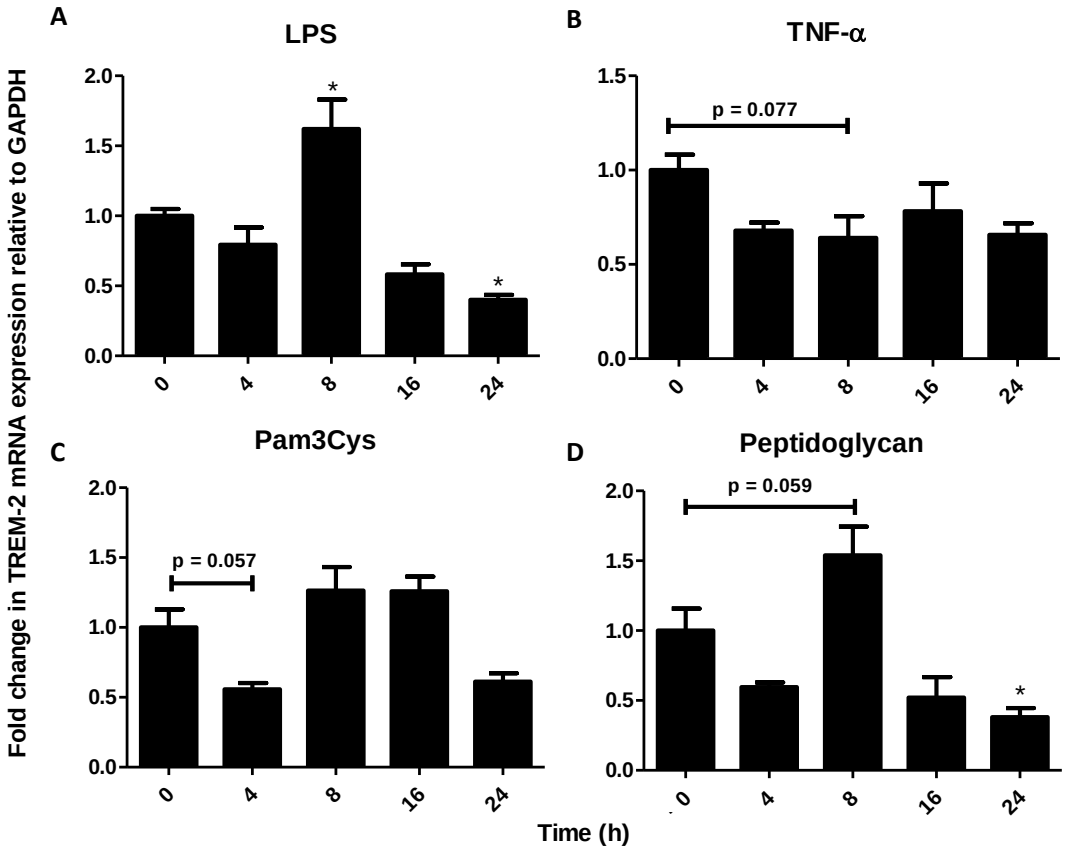

Figure 4.2: Regulation of TREM-2 mRNA expression by TNF-α and TLR agonists in THP-1 monocytes. THP-1 cells were incubated with (A) 10 µg/mL LPS (TLR4), (B) 10 ng/mL TNF-α, (C) 100 ng/mL Pam3Cys (TLR2), or (D) 10 µg/mL PGN (TLR2) for 4-24 h. TREM-2 mRNA expression was measured by qRT-PCR. mRNA values are expressed as fold change (relative to the control) normalised to GAPDH, mean ± SEM (n = 3), *$p \leq 0.05$ (one-way ANOVA with post hoc Dunnett's test).

4.3.2. TREM-2 Regulation by the Anti-inflammatory Mediators IL-4 and IL-13

IL-4 and IL-13 are increased in various inflammatory conditions, including MS, RA and COPD and can dampen down the inflammatory response in conditions, including pathogen infection and RA [447-451]. Since TREM-2 is also increased in these inflammatory conditions [242-244], this study investigated IL-4 and IL-13 for their ability to increase TREM-2 mRNA and protein expression in THP-1 monocytes. THP-1 cells were incubated for up to 24 h with 10 ng/mL IL-4 and TREM-2 mRNA expression measured by qRT-PCR. IL-4 increased TREM-2 mRNA expression in THP-1 monocytes between 4 and 24 h which was significant at 8 h ($p \leq 0.05$) (Figure 4.3A). Despite utilising the same receptor, IL-13 did not show a significant increase in TREM-2 mRNA expression, although there was a trend towards a small increase (Figure 4.3B).

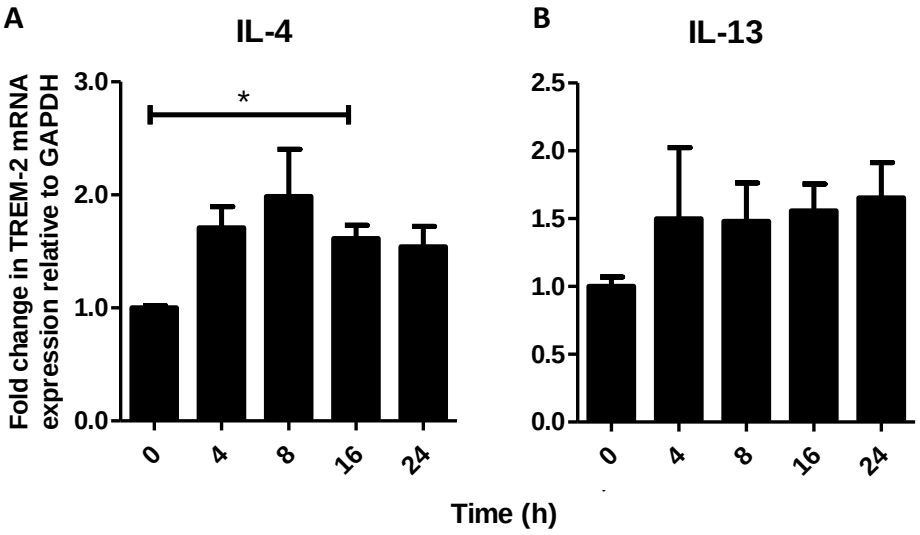

Figure 4.3: Regulation of TREM-2 expression by IL-4 and IL-13. THP-1 cells were incubated with (A) 10 ng/mL IL-4 or (B) 10 ng/mL IL-13 for 4-24 h. TREM-2 mRNA was measured by qRT-PCR. mRNA values are expressed as fold change relative to the control, normalised to GAPDH ± SEM (n = 3), *$p \leq 0.05$ (one-way ANOVA with post hoc Dunnett's test).

The effect of IL-4 on TREM-2 protein expression was also examined. Figure 4.4A demonstrates that IL-4 also induced TREM-2 protein expression in THP-1 monocytes, peaking at 8 h. Results were similar in serum-starved THP-1 cells, suggesting that growth factors in the serum that have previously been shown to increase TREM-2 expression (e.g. GM-CSF and M-CSF) were not required for this

change (Figure 4.4B). IL-4 also increased TREM-2 protein expression in primary human monocytes derived from peripheral blood, confirming the ability of IL-4 to induce TREM-2 expression in this cell type (Figure 4.4C).

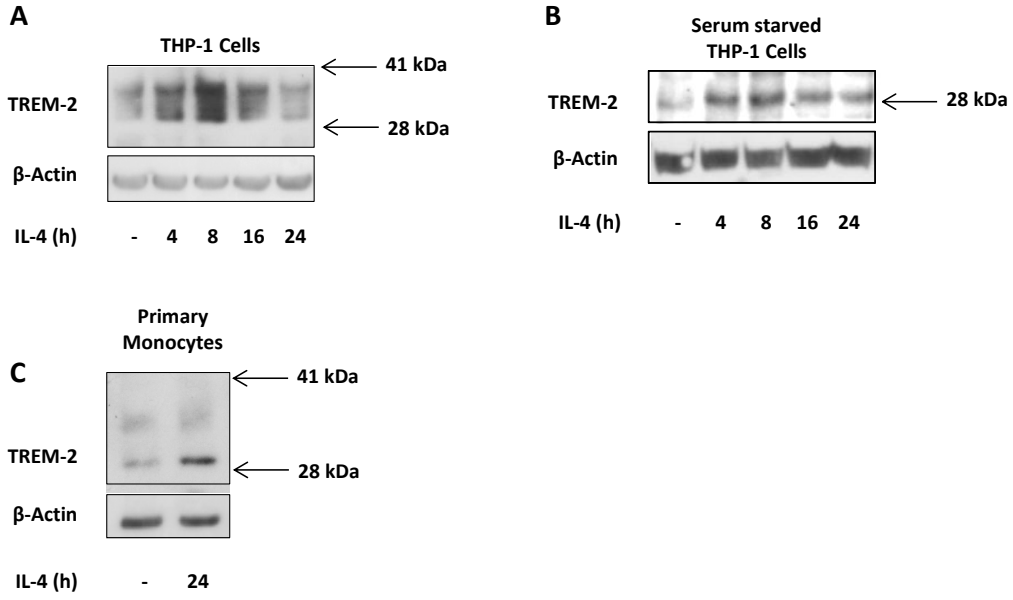

Figure 4.4: IL-4 increases TREM-2 protein expression in THP-1 cells. (A) THP-1 cells, (B) serum starved THP-1 cells or (C) primary monocytes isolated from peripheral blood were incubated with 10 ng/mL IL-4 for 4-24 h. TREM-2 protein expression was analysed by western blot analysis. β-actin was used as a loading control (n = 3).

In addition to its anti-inflammatory actions, IL-4 plays a role in differentiation of monocytes into other myeloid cell types including dendritic cells and macrophages [(452)]. In pilot experiments to investigate the effect of differentiation on IL-4-induced TREM-2 expression, THP-1 monocytes were differentiated into a macrophage-like cell line by treatment with 100 nM PMA for 72 h. IL-4 had no significant effect on TREM-2 mRNA expression in PMA-differentiated THP-1 cells (Figure 4.5A). However, at the protein level, IL-4 increased TREM-2 expression at 8, 16 and 24 h (Figure 4.5B). Incubation in culture also increased basal levels of TREM-2 following PMA differentiation suggesting that TREM-2 expression is increased further during the 48 h after PMA differentiation (Figure 4.5B). This suggests that IL-4 also increases TREM-2 expression in PMA-differentiated THP-1 cells but through a different mechanism that does not require *de novo* synthesis of TREM-2 mRNA.

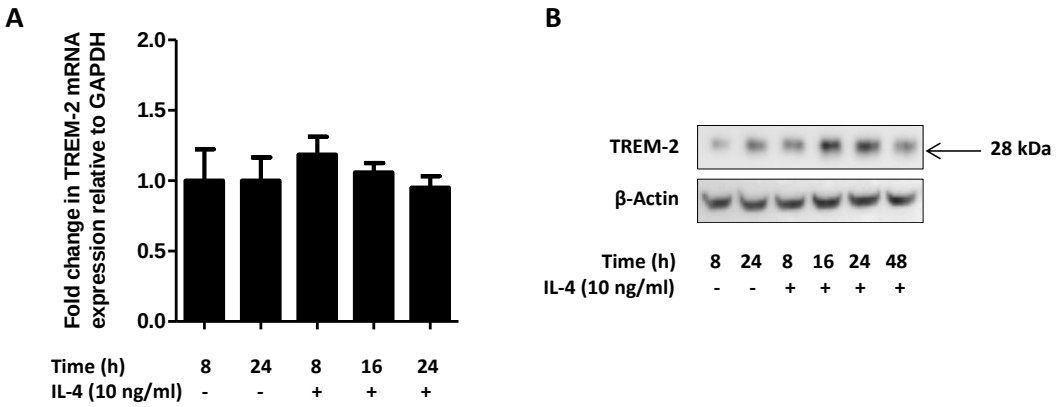

Figure 4.5: IL-4 increases TREM-2 expression in PMA-differentiated THP-1 cells. THP-1 cells differentiated with 100 nM PMA were stimulated with 10 ng/mL IL-4 for 8-48 h. (A) TREM-2 mRNA was measured by qRT-PCR. mRNA values are expressed as fold change (relative to the control) normalised to GAPDH ± SEM. (B) TREM-2 protein expression was measured by western blot analysis. β-actin was used as a loading control (n = 3).

4.3.3. IL-4-Induced TREM-2 Expression is STAT6 Independent

The mechanism by which IL-4 increases TREM-2 expression is currently unknown. STAT6 is the major signalling pathway required for IL-4 differentiation of monocytes/macrophages into M2-type macrophages [(444)]. TREM-2 has been identified as a marker of M2-type macrophages, suggesting that the STAT6 pathway may be the mechanism required for IL-4-induced TREM-2 expression [(102)]. The role of STAT6 in IL-4-induced TREM-2 expression was therefore investigated. STAT-6 siRNA was used to KD STAT6 expression in THP-1 cells. Cells were transfected with STAT6 siRNA or scrambled control for 24 h. Transfection with STAT6 siRNA resulted in a 61.9% KD of STAT6 mRNA expression analysed by RT-PCR ($p \leq 0.05$) (Figure 4.6A). STAT-6 siRNA had no effect on IL-4-induced TREM-2 mRNA (Figure 4.6B) or protein (Figure 4.6C) expression in THP-1 monocytes, suggesting that STAT6 is not required for IL-4-induced TREM-2 expression.

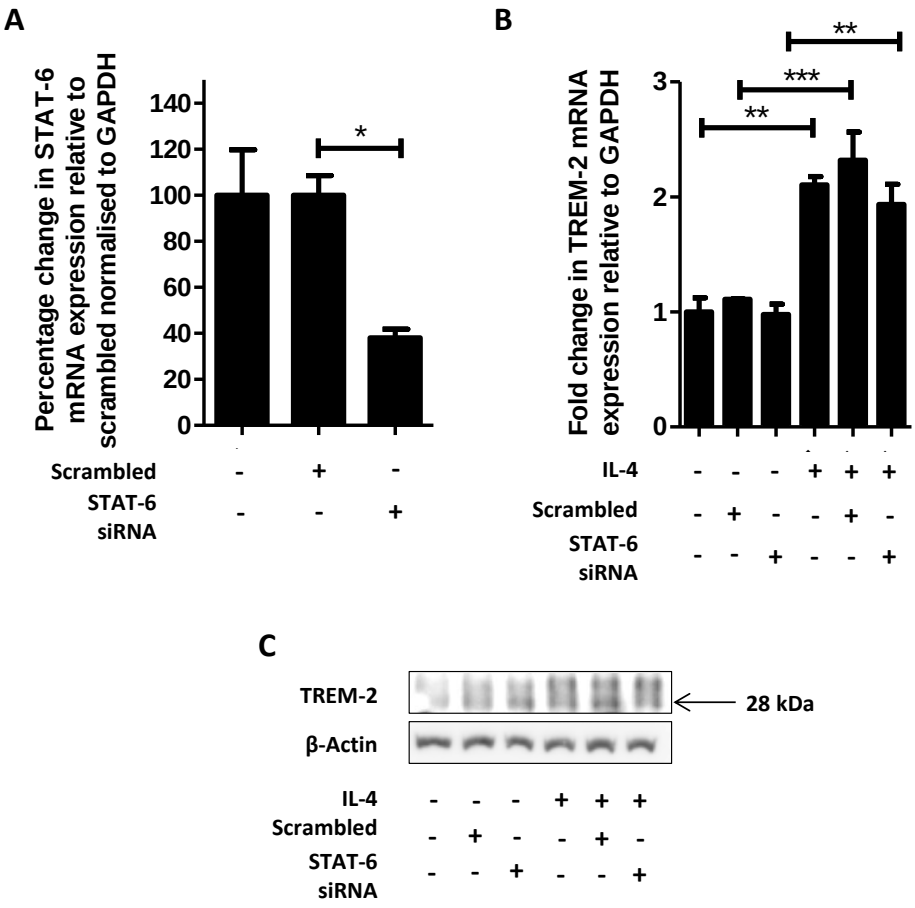

Figure 4.6: IL-4-induced TREM-2 expression is STAT-6 independent. THP-1 cells were non-transfected or transfected with 250 nM STAT6 siRNA or scrambled control for 24 h. A) STAT6 mRNA expression was measured 24 h after transfection. (B) 24 h after transfection, THP-1 cells were stimulated with IL-4 (10 ng/mL) for 8 h and TREM-2 mRNA expression was measured. mRNA was measured by qRT-PCR, values are expressed as % (A) or fold (B) change relative to the control, normalised to GAPDH ± SEM, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 (one-way ANOVA with post hoc Bonferroni test). (C) TREM-2 protein expression, measured by western blot analysis, following 8 h stimulation with IL-4 (10 ng/ml). β-actin was used as a loading control (n = 3).

4.3.4. Inhibition of PI3K Suppresses IL-4-Induced TREM-2 mRNA and Protein Expression

PI3K has recently emerged as an equally important factor as STAT6 in IL-4-induced alternative macrophage activation and therefore may play a role in IL-4-induced TREM-2 expression [453]. In addition to PI3K, the ERK signalling pathway is another STAT-6 independent pathway activated after IL-4 receptor activation [454]. To assess the effect of inhibition of the MEK/ERK1/2 and PI3K signalling pathways on IL-4-

induced TREM-2 expression, THP-1 cells were pre-incubated with inhibitors for 30 min prior to stimulation with IL-4 for 8 h. PD98059 (10 μM) had no effect on IL-4-induced TREM-2 mRNA expression, suggesting that the ERK1/2 signalling pathway is not involved in IL-4-induced TREM-2 mRNA expression (Figure 4.7A). However, 10 μM LY294002 resulted in a significant inhibition ($p \leq 0.01$) of IL-4-induced TREM-2 mRNA expression (Figure 4.7B). Furthermore, this effect was dose-dependent (Figure 4.7C), suggesting that PI3K is required for IL-4-induced TREM-2 mRNA expression. These inhibitors were also assessed for their cytotoxicity by MTS assay. The ERK1/2 signalling pathway inhibitor PD98059 and PI3K inhibitor LY294002 were not toxic at 5 or 10 μM but cell viability was reduced by 22.6% and 13.8% at 20 μM respectively (see Appendix Figure 8.1).

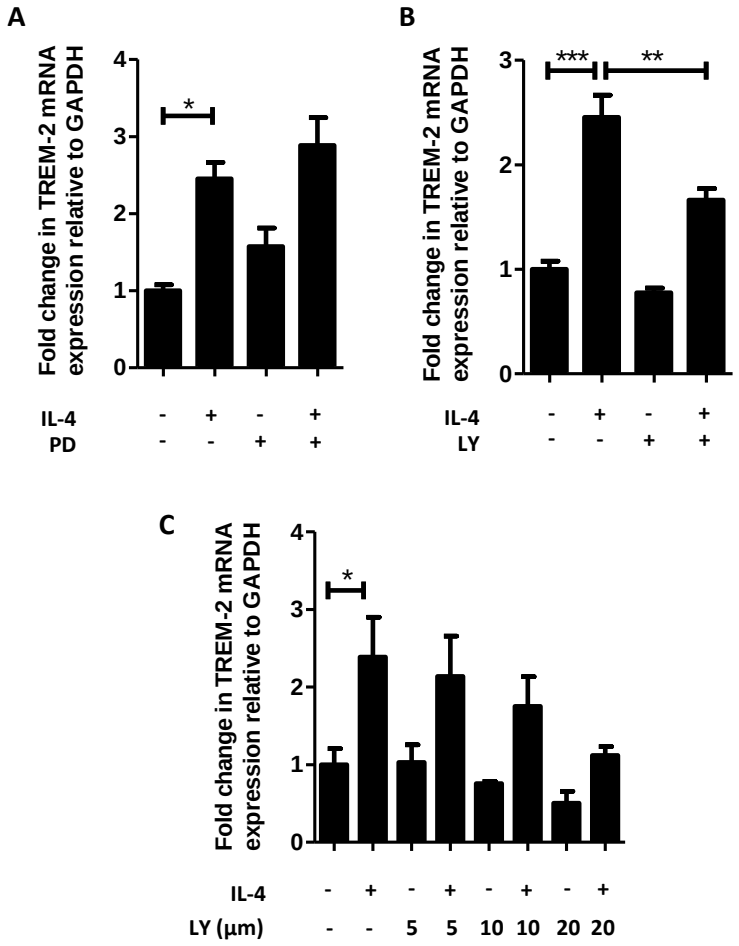

Figure 4.7: Inhibition of PI3K suppresses IL-4-induced TREM-2 mRNA expression. THP-1 cells were pre-incubated with (A) 10 μM PD98059 (PD) or (B) LY294002 (LY) 30 min prior to stimulation with 10 ng/mL IL-4 for 8 h. TREM-2 mRNA expression was measured by qRT-PCR. (C) THP-1 cells were pre-incubated with 5, 10 or 20 μM LY294002 30 min prior to 8 h stimulation

with IL-4 (10 ng/mL). TREM-2 mRNA expression was measured by qRT-PCR. mRNA values are expressed as fold change (relative to the control) normalised to GAPDH ± SEM, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 (one-way ANOVA with post hoc Bonferroni test) (n = 3).

Similarly, PD98059 had little effect on IL-4-induced TREM-2 protein but slightly reduced basal TREM-2 expression (Figure 4.8). Incubation with the PI3K inhibitor LY294002 corresponded to the mRNA results, showing a reduction in IL-4-induced TREM-2 protein expression (Figure 4.8). Similarly to the effect of the ERK1/2 inhibitor on TREM-2 protein expression alone, incubation with the PI3K inhibitor LY294002 also reduced basal TREM-2 protein expression. Although this image was representative of each of the biological replicates showing a consist suppression of IL-4 induced TREM-2 expression with LY294002, doing densitometry on the blots showed too much variation to produce a significant change. These results suggest that IL-4 activates PI3K to induce *de novo* synthesis of TREM-2 mRNA and therefore increase TREM-2 protein expression.

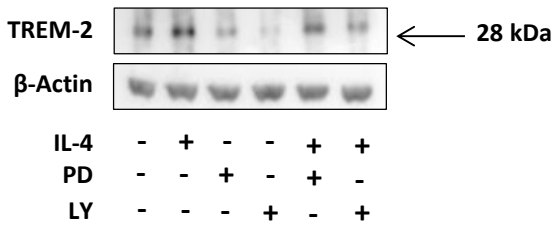

Figure 4.8: PI3K reduces IL-4-induced TREM-2 protein expression. THP-1 cells were pre-incubated with 10 μM MEK1/ERK1/2 signalling pathway inhibitor PD98059 (PD) or the PI3K inhibitor LY294002 (LY) 30 min prior to stimulation with IL-4 (10 ng/mL) for 8 h. TREM-2 protein expression was measured by western blot analysis. β-actin was used as a loading control (n = 3).

4.4. Discussion

TREM-2 is increased in patients and animal models of chronic inflammatory disease including COPD, diabetes and MS [242, 243, 364]. However, current knowledge of TREM-2 suggests that in macrophages, dendritic cells, microglial cells and endothelial cells, pro-inflammatory mediators actually reduce TREM-2 expression [81, 240, 241]. This study reported that TLR 2 and 4 agonists modulate TREM-2 expression in THP-1 cells for the first time. More interestingly, IL-4 an important mediator present in inflammatory conditions including MS, arthritis and COPD [448, 449, 455] was found to induce TREM-2 expression in monocytic cells via a PI3K dependent, STAT6-independent pathway. These data suggest that IL-4 may be, in part, responsible for the increase in TREM-2 expression observed in inflammatory conditions and shows for the first time the role of PI3K in IL-4-induced TREM-2 expression.

In this study, THP-1 monocytes, previously characterised in the laboratory as a model of monocytes, were used to study the effect of pro- and anti-inflammatory mediators on TREM-2 expression. Suppression of TREM-2 expression by pro-inflammatory mediators including TNF-α and LPS has been previously observed in hepatic macrophages, endothelial cells and microglia [240, 241, 243]. In this study, a similar effect was seen in a murine monocyte-derived macrophage cell line and in THP-1 monocytes. In addition, a biphasic regulation of TREM-2 by TLR 2 and 4 agonists was seen at the mRNA level in monocytes with TREM-2 expression being reduced at 4 and 24 h, but returned to baseline levels, or in some cases increased, by 8 h. This suggests that there may be multiple pathways regulating TREM-2 expression following TLR stimulation. This may be, in part, via NFκB, as it is a key signalling pathway activated after TLR activation. In addition, VIP has been shown to reduce LPS-induced TREM-2 supression, which is likely to occur via its ability to inhibit NFκB binding to DNA [374]. In addition, TLR2 and 4 agonists show very similar kinetics for regulating TREM-2 mRNA expression and therefore are also likely to signal via similar pathways. TNF-α on the other hand, demonstrated different kinetics, with a non-significant reduction in TREM-2 expression and no differences between the time points observed, suggesting that TNF-α may not be acting in the same way as the TLR agonists to regulate TREM-2 expression.

IL-4 is important for differentiation of monocytes and macrophages into M2-type macrophages. M2-type macrophages are known for their anti-inflammatory properties reducing the secretion of pro-inflammatory cytokines and increasing the

secretion of anti-inflammatory cytokines, resulting in the suppression of the inflammatory response [46]. More recently, TREM-2 has been used as a marker of M2 macrophage activation, due to its high expression on this cell type [303-305]. Taken together, this suggests that TREM-2 may have an important function in this cell type by mediating some of the anti-inflammatory properties of M2-type macrophages. IL-4 in combination with either M-CSF or GM-CSF has previously been shown to induce TREM-2 expression in monocytes by inducing differentiation into M2-type macrophages and dendritic cells [336]. However, M-CSF has been previously shown to increase TREM-2 expression [240]. In this study, IL-4 increased TREM-2 expression independently of M-CSF or other growth factors in THP-1 cells and primary monocytes which is supported by a study from Cella and colleagues who showed that IL-4 alone increased TREM-2 expression in $CD14^+$ monocytes [299]. This also suggests that there may be other functions for IL-4-induced TREM-2 expression other than as a marker of differentiation. Interestingly, this induction of TREM-2 was observed at a later time point in primary monocytes. This may be due to the fact that primary monocytes derived from peripheral blood start to differentiate in culture, which may alter the kinetics of IL-4-induced TREM-2 expression. On the other hand, in allergy, IL-4 is a pro-inflammatory cytokine, increasing Th2 cell proliferation and inducing class-switching to IgE, increasing airway remodelling and promoting allergic inflammation [456]. TREM-2 is also upregulated during allergic inflammation, in the ovalbumin model of allergy in dendritic cells and in the murine house dust mite model of airway inflammation [240, 367]. This study shows that IL-4, which is highly expressed in allergic inflammation, increases TREM-2 expression, suggesting that IL-4 may be a key player in the induction of TREM-2 expression in allergy. In contrast, using PMA-differentiated THP-1 cells as a model of macrophages, IL-4 had no effect on TREM-2 mRNA expression, but increased TREM-2 protein expression suggesting that IL-4 increases TREM-2 expression post-translationally in these cells. Therefore, IL-4 increases TREM-2 expression in THP-1 cells and PMA-differentiated THP-1 cells through different mechanisms, suggesting that differentiation of these cells alters the mechanisms of TREM-2 regulation.

IL-4 and IL-13 signal through a shared receptor, and therefore have similar actions to each other [107]. This study also investigated the effect of IL-13 on TREM-2 expression in THP-1 cells. In contrast to IL-4, IL-13 had no significant effect on TREM-2 mRNA expression, although there was a trend that suggested a small increase in TREM-2 at all time points studied with similar kinetics to IL-4. This is

likely to be due to different binding affinities of IL-13 and IL-4 to the receptors expressed. IL-4 and IL-13 can bind to IL-4R(α)/IL-13R(α)1 receptor complex, but IL-4 can also bind to the IL-4R(α)/γc receptor complex [457]. This suggests that IL-4 alone has more available receptors for activation and signal transduction in this system compared to IL-13, which may be why very little response was observed with IL-13 alone [458].

There is very little known about the signalling pathways that regulate TREM-2 expression. This area was a key focus due to the lack of current understanding and the emerging importance of increased TREM-2 expression in inflammatory diseases. STAT6 is a major signalling mediator for IL-4, particularly in IL-4-induced alternative activation. However, in this study, IL-4-induced TREM-2 was independent of STAT6. In support of our findings, Turnbull and co-workers demonstrated that the increase in TREM-2 induced by thioglycollate inflammation was independent of STAT6 signalling [240].

More recently, PI3K has also been identified as an important mediator in alternative activation [453, 459]. Here, the PI3K inhibitor LY294002 suppressed IL-4-induced TREM-2 expression, suggesting that PI3K is important for its regulation. PI3K is an important signalling protein in inflammation and the immune response including leukocyte migration and B- and T-cell activation [460]. Activation of the AKT/PI3K signalling pathway suppresses LPS signalling, leading to a reduction in pro-inflammatory cytokine secretion [461]. TREM-2 also suppresses LPS signalling and therefore PI3K may increase TREM-2 expression for its inhibitory effects on LPS-induced cytokine secretion. In addition to the effects of PI3K inhibition on induced TREM-2 expression, the PI3K inhibitor LY294002 also suppressed basal levels of TREM-2 protein expression, although no significant effect was observed at the mRNA level. This effect has also been observed by Chen and co-workers in hepatic macrophages and endothelial cells [241]. However, Chen and co-workers showed that suppression of TREM-2 expression observed by IL-1β and TNF-α was not through the PI3K signalling pathway [241]. Interestingly, this study found that PI3K not only regulates basal expression of TREM-2 but also regulates induced TREM-2 expression by IL-4.

Future studies will investigate the transcription factor and other members of the signalling pathway required for IL-4-induced TREM-2 expression. The PI3K/AKT signalling pathway inhibits GSK3 [462]. Active GSK3 inhibits transcription factor activity including GATA-4, NFATc and β-catenin and therefore these transcription

factors are targets for future studies of the IL-4-induced TREM-2 expression [463-465]. Future studies also aim to investigate the effect of other inflammatory mediators on TREM-2 expression including prostaglandins, chemokines and a variety of cytokines not studied here including IL-17, IL-22, IL-10, and IL-6 and to confirm regulation of TREM-2 in primary cells.

4.5. Summary

This study has identified IL-4 as an inducer of TREM-2 expression in monocytes. As IL-4 is increased in many inflammatory conditions, IL-4 is likely to contribute to the increased TREM-2 expression observed in conditions including MS and asthma. In addition, IL-4-induced TREM-2 expression was independent of STAT6, a major IL-4 signalling pathway, but dependent on PI3K. These results demonstrate for the first time, the role of PI3K in IL-4-induced TREM-2 regulation.

5. Regulation of TREM-2 Expression by TGF-β1 in Myeloid Cells

Acknowledgements:

- Florine Squirrell, MPharm undergraduate project student, performed the following experiments:
    - Figure 5.1 TGF-β1-induced TREM-2 protein (n = 1).
    - Figure 5.4A (n = 1) and Figure 5.6A (n = 2) SB, PD inhibitor experiments.
- SMAD3 KD and SMAD3 mRNA expression (Figure 5.7 and 5.8) were performed by Tom Davies from Dipak Ramji's laboratory at the University of Cardiff.

5.1. Introduction

TGF-β1 is another cytokine known for its ability to resolve inflammation. TGF-β1 is increased in inflammatory conditions including COPD and MS and in wound healing [466-468]. In MS and wound healing, TGF-β1 has demonstrated beneficial effects [119, 469]. These effects of TGF-β1 are due to its role in tissue repair and regeneration [119, 238, 470]. TGF-β1 KO mice demonstrate inefficient wound healing following injury [119]. TGF-β1 mediates its repair functions by encouraging epithelial migration and regulation of the extracellular matrix [466, 471]. One of the main ways TGF-β1 regulates the extracellular matrix is through the regulation of MMPs. MMPs are a family of proteases that degrade extracellular matrix proteins including fibronectin, collagen and gelatin [472]. TGF-β1 induces and suppresses MMPs both directly and indirectly through the regulation of inhibitors for example tissue inhibitor of metalloproteinases (TIMPs) [473-475]. The regulation of these proteases is thought to be important for the regeneration and repair functions of TGF-β1. TGF-β1 is also important for dampening down the immune response by reducing Th1 type T-cell responses and pro-inflammatory cytokines such as IL-12 and increasing regulatory T-cell activity and anti-inflammatory cytokines such as IL-10, driving the resolution of inflammation. These anti-inflammatory functions are another reason for its beneficial effects in wound healing and diseases including MS and stoke [119, 476, 477].

These functions of TGF-β1 require the activation a variety of signalling pathways. The two most characterised are the SMAD and MAPK signalling pathways. TGF-β1 activates the TGF-β receptor I/II dimer, initiating phosphorylation of SMAD2 and/or SMAD3 [478]. Classically, the SMAD2/3 complex dimerises with SMAD4 and is transported to the nucleus where it binds to SMAD response elements (SRE) on gene promoters altering gene expression [130]. However, TGF-β1 also signals independently of SMADs activating signalling molecules including TAK-1, Ras and Raf and PI3K [131, 132]. Phosphorylation of Ras and Raf leads to the activation of the MEK1/ERK1/2 signalling pathway which regulates some of the functions of TGF-β1 in fibrosis and migration [479, 480]. In addition, TGF-β-induced phosphorylation of TAK-1 leads to the activation of several other kinases including p38 and JNK which signal independently to induce a variety of different functions of TGF-β [133, 134, 481].

TREM-2 is important for resolution of inflammation and in tissue repair. TREM-2 is also increased in many inflammatory conditions, however there are still only a few mediators known to increase its expression [242, 243, 334]. Chapter 3 showed that the anti-inflammatory cytokine IL-4 increases TREM-2 expression. Due to the role of

TGF-β1 in the tissue repair and the resolution of inflammation [119, 476], this cytokine was investigated for its effects on TREM-2 expression along with the mechanisms required for its regulation of TREM-2.

5.2. Aims

The aims of this study were to:

- Investigate the effect of TGF-β1 on TREM-2 expression in primary monocytes, monocyte and macrophage cell lines.
- Study the mechanisms of TREM-2 regulation by TGF-β1 in the THP-1 monocyte cell line.
- Explore the functional role of TGF-β1-induced TREM-2 in THP-1 monocytes.

5.3. Results

5.3.1. TGF-β1 Increases TREM-2 mRNA and Protein Expression in THP-1 cells and Primary Monocytes

The effects of TGF-β1 on TREM-2 expression in monocytes and macrophage cell lines were investigated. TREM-2 mRNA and protein expression were measured by qRT-PCR and western blot, respectively. In the presence of TGF-β1, TREM-2 mRNA expression was increased, peaking at 16 h in THP-1 cells ($p \leq 0.05$) (Figure 5.1A). TGF-β1 also increased TREM-2 expression at the protein level, peaking at 48 h (Figure 5.1B). This experiment was also performed in primary monocytes derived from peripheral blood which also showed an increase in TREM-2 following TGF-β1 stimulation, confirming that this signalling pathway in THP-1 cells is also present in primary monocytes (Figure 5.1C).

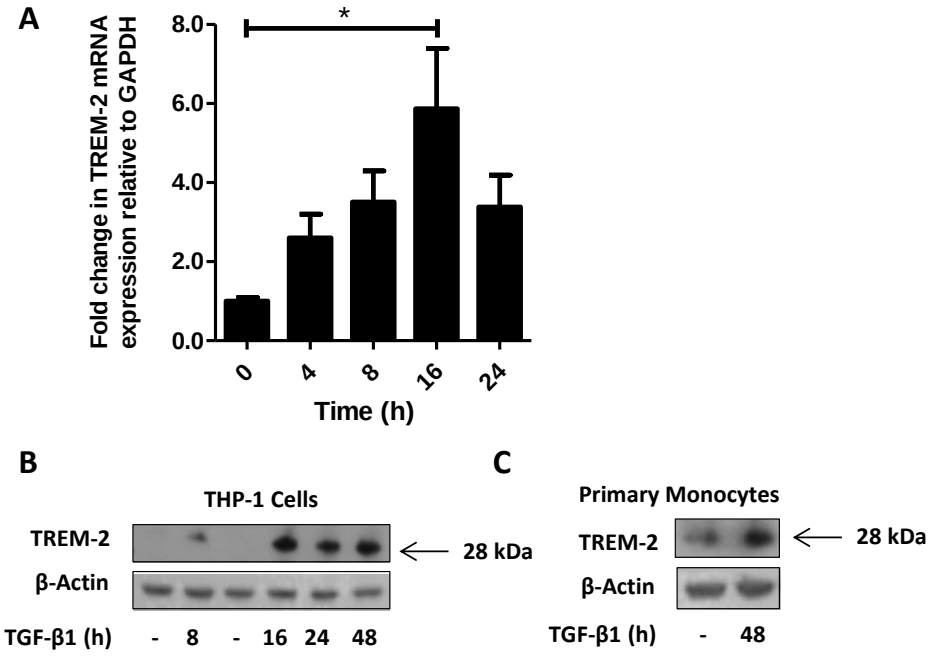

Figure 5.1: TGF-β1 increases TREM-2 expression in THP-1 cells and primary monocytes. (A) THP-1 cells were incubated with or without 5 ng/mL TGF-β1 for 4-24 h and TREM-2 mRNA expression measured by qRT-PCR. mRNA values are expressed as fold change relative to the 0 h control normalised to GAPDH ± SEM, $*p \leq 0.05$ (one-way ANOVA with post hoc Dunnett's test). (B) THP-1 cells or (C) primary monocytes were stimulated with or without TGF-β1 (5 ng/mL) for 8-48 h and TREM-2 protein expression measured by western blot analysis. β-actin was used as loading control (n = 3).

To analyse the kinetics of TGF-β1-induced TREM-2 protein expression, TGF-β1 was added to THP-1 cells for up to 7 days. The increase in TREM-2 protein induced by TGF-β1 remained high up to 7 days of stimulation, suggesting that this increase in TREM-2 may be permanent (Figure 5.2A). However, removing TGF-β1 after 48 h reduced TREM-2 expression at 6 days, showing that the change in TREM-2 protein expression after TGF-β1 can be reversed by removing TGF-β1. This suggests that this increase in TREM-2 is not due to a permanent change in cell type (Figure 5.2B).

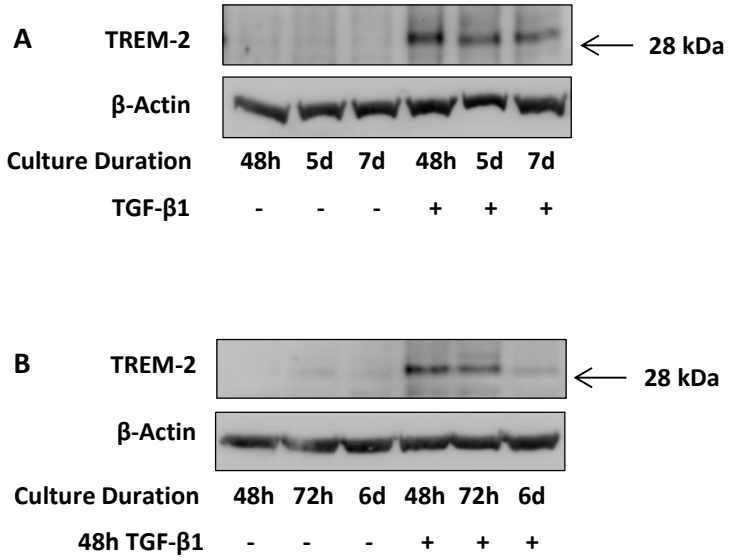

Figure 5.2: Upregulation of TREM-2 by TGF-β1 is not a permanent change. THP-1 cells were incubated with or without 5 ng/mL TGF-β1 for (A) 48 h, 5 days (d) or 7 days or (B) 48 h, 72 h or 6 days. In (B) samples cultured longer than 48 h had TGF-β1 removed at 48 h. TREM-2 protein expression was measured by western blot analysis. β-actin was used as loading control (n = 3).

5.3.2. *TGF-β1 had no Effect on TREM-2 Expression in PMA-Differentiated THP-1 Cells*

TGF-β1 also exhibits important functions in macrophages including suppression of inflammation by reducing pro-inflammatory cytokine secretion [(475)]. To assess the effect of TGF-β1 on TREM-2 expression in macrophages, this study used the murine macrophage cell line RAW 264.7 and PMA-differentiated THP-1 cells to measure TREM-2 mRNA and protein expression. Unlike monocytes, stimulation of

RAW264.7 macrophages had no effect on TREM-2 mRNA expression (Figure 5.3A). Similar results were also seen in the PMA-differentiated THP-1 cells (Figure 5.3B). In addition, TGF-β1 did not increase TREM-2 protein expression in PMA-differentiated THP-1 cells, indeed it seemed to decrease it at 48 h (Figure 5.3C).

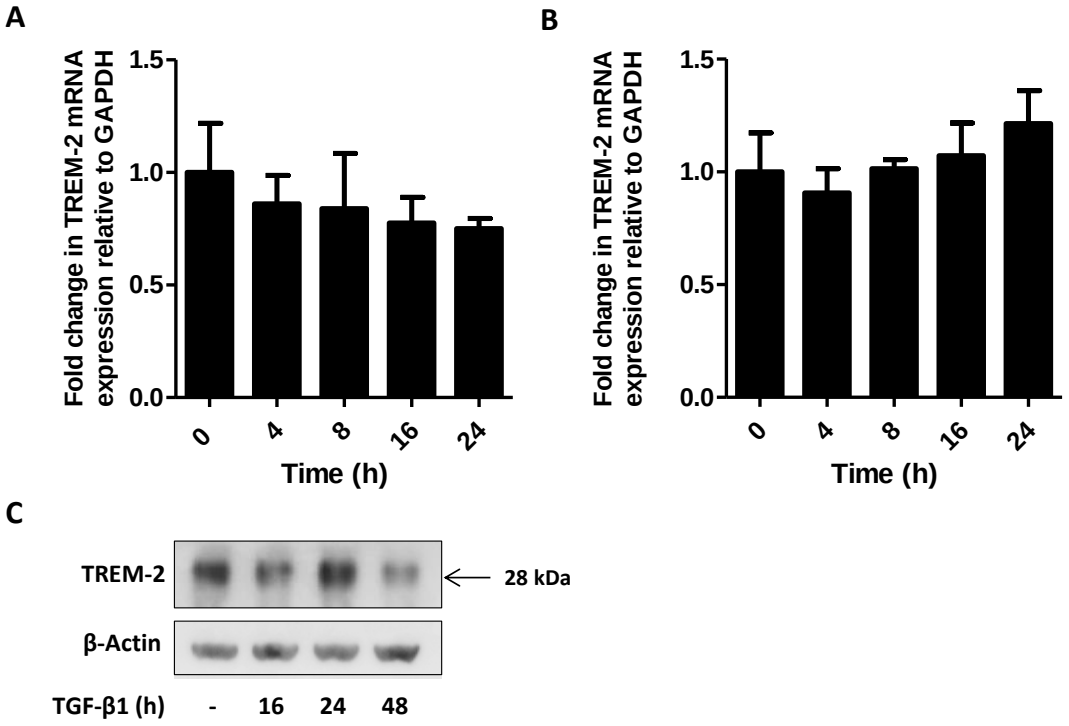

Figure 5.3: Effect of TGF-β1 on TREM-2 mRNA and protein expression in RAW 264.7 and PMA-differentiated THP-1 cells. (A) RAW 264.7 cells or (B) PMA-differentiated THP-1 cells were incubated with or without 5 ng/mL TGF-β1 for 4-24 h and TREM-2 mRNA expression measured by qRT- PCR. mRNA values are expressed as fold change relative to the control, normalised to GAPDH, mean ± SEM (n = 3). (C) PMA-differentiated THP-1 cells were stimulated with or without 5 ng/mL TGF-β1 for 16-48 h and TREM-2 protein measured by western blot analysis (n = 2). β-actin was used as a loading control.

5.3.3. TGF-β1-Induced TREM-2 Expression Requires p38 and ERK1/2

TGF-β1 has been shown to activate several kinase pathways including PKC, PI3K and MAPK [132, 133, 482]. The effect of the inhibitors of p38 MAP kinase (SB203580), PI3K (LY294002), the ERK1/2 signalling pathway (PD98059) and PKC (bisindolylmaleimide I) on TGF-β1-induced TREM-2 mRNA expression were then studied. SB203580 significantly reduced TGF-β1-induced TREM-2 mRNA expression at 16 h ($p \leq 0.001$) (Figure 5.4A). A small but significant reduction in TGF-β1-induced TREM-2 mRNA was also observed in the presence of LY294002 ($p \leq 0.01$) (Figure 5.4C). In contrast, PD98059 and bisindolylmaleimide I had no effect on TGF-β1-induced TREM-2 mRNA expression (Figure 5.4A and B). SB203580 showed a trend of dose-dependent inhibition of TGF-β1-induced TREM-2 mRNA expression (not significant) (Figure 5.4D). Together, this data suggests that p38 MAP kinase and PI3K are important for TGF-β1-induced TREM-2 mRNA expression. The effects of the inhibitors on cell viability were tested by MTS assay to ensure that they were not cytotoxic at the concentrations used in this study. THP-1 cells were incubated with inhibitors for 16 h and cell viability was then measured. The p38 MAP kinase and PKC inhibitors SB203580 (SB) and bisindolylmaleimide I (BIS) were not toxic at any concentration (see Appendix Figure 8.2). In addition, Figure 8.1 (see Appendix) demonstrates that the MEK1/ERK1/2 and PI3K signalling pathway inhibitors PD98059 (PD) and LY294002 (LY) (respectively) were not toxic up to 10 µM so this concentration was used for both inhibitors.

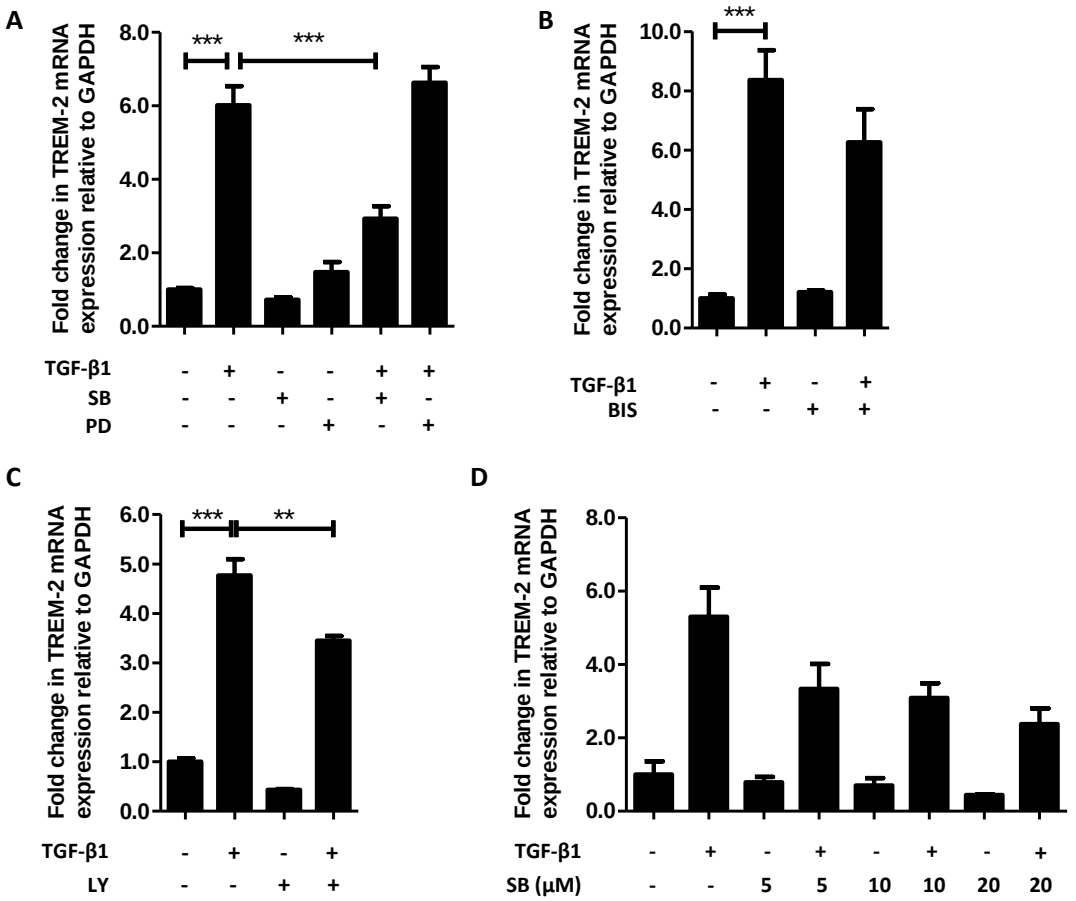

Figure 5.4: p38 MAP kinase and PI3K regulate TGF-β1-induced TREM-2 mRNA expression. THP-1 cells were pre-incubated for 30 min with (A) 10 μM SB203580 (SB) or 10 μM PD98059 (PD), (B) 10 μM bisindolylmaleimide I (BIS) or (C) 10 μM LY294002 (LY) followed by 16 h treatment with 5 ng/mL TGF-β1 and TREM-2 mRNA measured by qRT-PCR. (D) THP-1 cells were incubated with or without 5 ng/mL TGF-β1 in the presence or absence of 5 μM, 10 μM or 20 μM SB for 16 h. mRNA values are expressed as fold change relative to the control, normalised to GAPDH ± SEM, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ (one-way ANOVA with post hoc Bonferroni test) (n = 3).

To confirm the effects of these inhibitors on TREM-2 expression at the protein levels, these inhibitors were analysed for their effects on TGF-β1-induced TREM-2 protein expression measured by western blot analysis. The PKC inhibitor, bisindolylmaleimide I, had no effect on TGF-β1-induced TREM-2 expression (Figure 5.5A). Similarly to the observation at the mRNA level, the PI3K inhibitor LY294002 reduced TGF-β1-induced TREM-2 protein expression (Figure 5.5B).

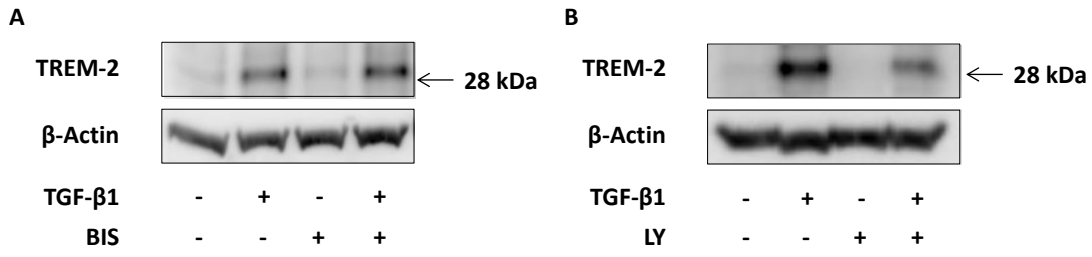

Figure 5.5: The effect of PKC and PI3K inhibition on TGF-β1-induced TREM-2 protein expression. THP-1 cells pre-incubated for 30 min with (A) 10 µM bisindolylmaleimide I (BIS) or (B) 10 µM LY294002 (LY) followed by 48 h treatment with 5 ng/mL TGF-β1 and TREM-2 protein expression measured by western blot analysis. β-actin was used as a loading control (n = 3).

In agreement with the observations at the mRNA level, SB203580 also reduced TGF-β1-induced TREM-2 protein expression (Figure 5.6A). However, in contrast to the effects of PD98059 at the mRNA level, PD98059 almost completely abolished TGF-β1-induced TREM-2 protein expression, suggesting that this signalling pathway is required post-translationally for TGF-β1-induced TREM-2 protein expression (Figure 5.6A). TGF-β1-induced p38 MAP kinase activation was confirmed by investigating p38 MAP kinase phosphorylation by western blot analysis. TGF-β1-induced p38 phosphorylation after 1 h of stimulation in THP-1 cells (Figure 5.6B). The effect of these inhibitors on cell viability was measured over 48 h to confirm the effects observed were not due to cytotoxicity. THP-1 cells incubated with bisindolylmaleimide I or SB203580 for 48 h did not demonstrate reduction in cell viability (see Appendix Figure 8.3). PD98059 reduced cell viability by 28.3%, 33.2% and 33.2% at 5, 10 and 20 µM respectively and LY294002 by 29.4%, 27.3% and 36.4% at 5, 10 and 20 µM respectively (see Appendix Figure 8.3). However, this small reduction in cell viability observed with LY294002 and PD98059 are unlikely to affect these results. Together, these experiments suggest that PI3K and the p38 and ERK1/2 MAP kinase pathways are important for TGF-β1-induced TREM-2 expression.

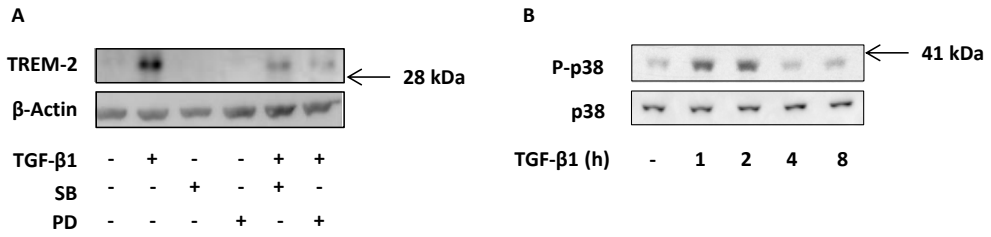

Figure 5.6: The p38 and ERK1/2 MAP kinase signalling pathways are required for TGF-β1-induced TREM-2 protein expression. (A) THP-1 cells were pre-incubated for 30 min with 10 µM SB203580 (SB) or PD98059 (PD) followed by 48 h treatment with 5 ng/mL TGF-β1 and TREM-2 protein expression measured by western blot analysis. (B) THP-1 cells were stimulated with 5 ng/mL TGF-β1 for 1-8 h and phosphorylated p38 (P-p38) and basal p38 protein expression measured in by western blot analysis. β-actin and p38 were used as loading controls for TREM-2 (A) and (B) P-p38 respectively (n = 3).

5.3.4. TGF-β1-Induced TREM-2 Expression is Independent of SMAD3, ATF2 and PPARγ

Several transcription factors are known to be activated by TGF- β1, including the SMAD family, ATF2 and PPARγ [130, 482, 483]. SMAD3 is central to SMAD signalling as it binds to SMADs 2 and 4 and also cross-talks with other signalling pathways including the p38 MAP kinase pathway [484, 485]. Since p38 MAP kinase is also required for TGF-β1-induced *de novo* synthesis of TREM-2 mRNA, the effects of SMAD3 on TGF-β1-induced TREM-2 expression was examined using SMAD3 siRNA. THP-1 cells transfected with SMAD3 siRNA resulted in a 62.1% KD in SMAD3 mRNA expression and complete inhibition of SMAD3 protein when compared with scrambled control (Figure 5.7 A and B).

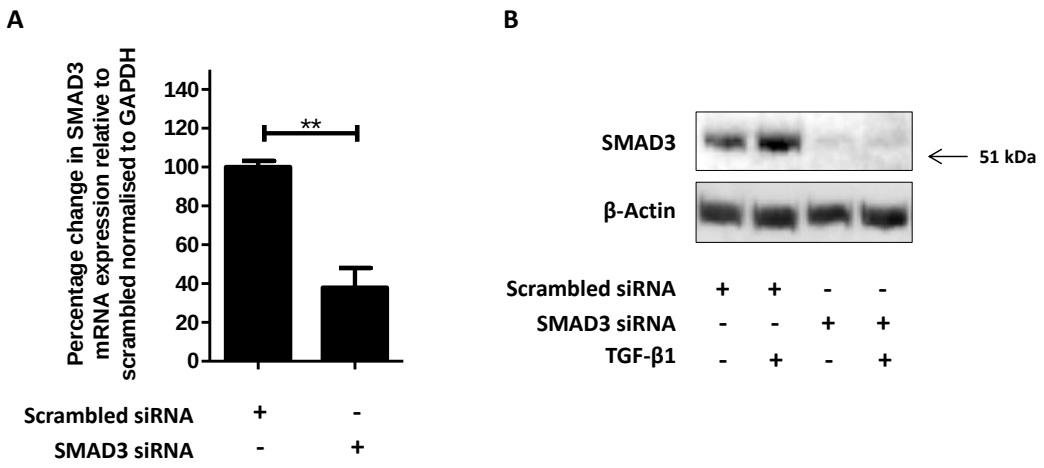

Figure 5.7: siRNA KD of SMAD3 in THP-1 cells. THP-1 cells were transfected with 9.55 nM SMAD3 siRNA or scrambled control for 24 h. 24 h after transfection, the cells were incubated for a further 16 h (A) alone or (B) with or without 5 ng/mL TGF-β1. SMAD3 (A) mRNA or (B) protein was measured by qRT-PCR and western blot analysis respectively. mRNA values are expressed as % change relative to the control and normalised to GAPDH ± SEM, *p ≤ 0.05 (unpaired Student's t test). For western blot analysis β-actin was used as a loading control (n = 3).

SMAD3 siRNA had no effect on TGF-β1-induced TREM-2 mRNA or protein expression (Figure 5.8A and B). Together, these results suggest that TGF-β1-induced TREM-2 expression is independent of SMAD3 signalling.

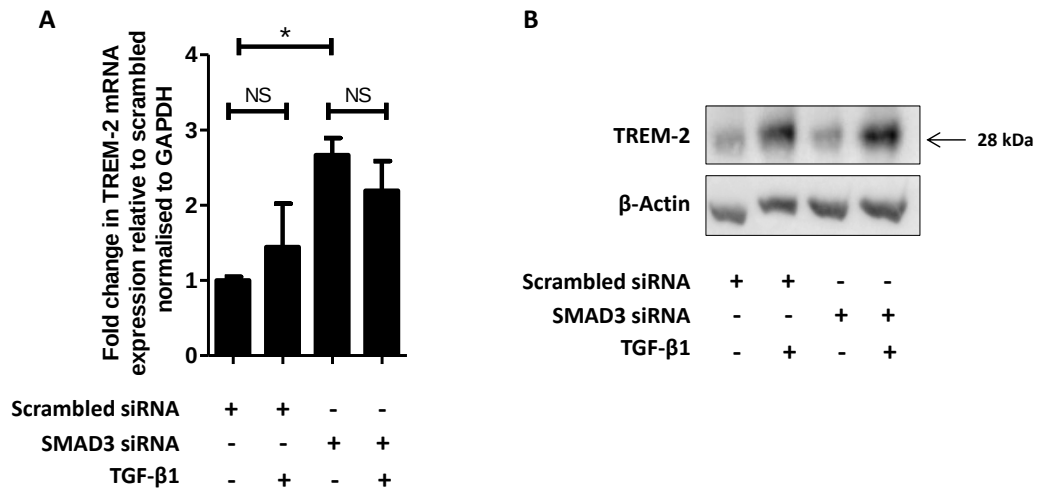

Figure 5.8: TGF-β1-induced TREM-2 expression is independent of SMAD3. THP-1 cells were transfected with 9.55 nM SMAD3 siRNA or scrambled control for 24 h. 24 h after transfection, the cells were stimulated with 5 ng/mL TGF-β1 for a further (A) 16 h for TREM-2 mRNA or (B) 48 h for TREM-2 protein. mRNA expression was measured by qRT-PCR, values are expressed as fold change relative to the control, normalised to GAPDH ± SEM, *p ≤ 0.05 (one-way ANOVA with post hoc Bonferroni test). TREM-2 protein expression was measured by western blot analysis. β-actin was used as a loading control (n = 3). NS, not significant.

The transcription factor ATF-2 is another key regulator of the TGF-β1 signalling pathway. ATF2 is increased following TGF-β1-induced p38 activation, and alters gene expression by binding to cyclic adenosine monophosphate (cAMP) response elements in gene promoters [(482)]. ATF-2 siRNA was therefore used to investigate the role of ATF-2 in TGF-β1/p38-induced TREM-2 expression. ATF-2 siRNA reduced ATF2 mRNA expression at 24 h by 60.8% (Figure 5.9A). ATF-2 siRNA had no effect on TREM-2 expression induced by TGF-β1 at either the mRNA (Figure 5.9B) or protein (Figure 5.9C) level. This study demonstrates that TGF-β1-induced TREM-2 expression is also independent of ATF2.

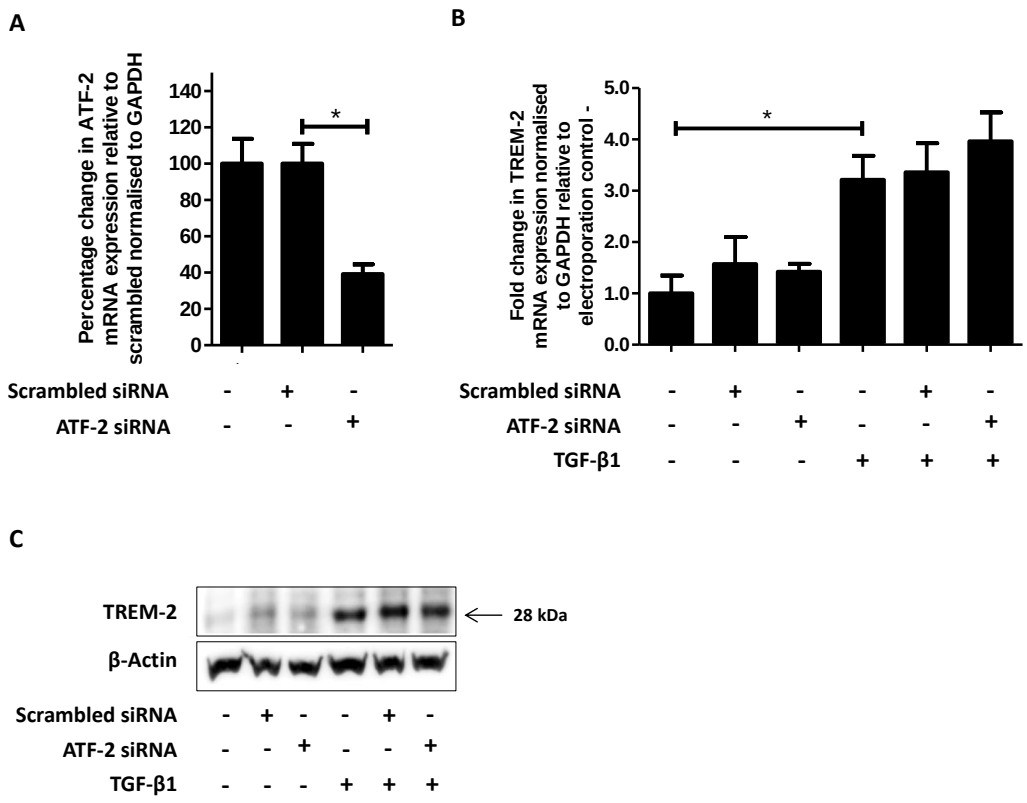

Figure 5.9: TGF-β1-induced TREM-2 expression is independent of the transcription factor ATF-2. THP-1 cells were non-transfected or transfected with 250 nM ATF-2 siRNA or scrambled control for 24 h. (A) ATF-2 mRNA expression measured 24 h after transfection. Alternatively, 24 h after transfection, the cells were stimulated with 5 ng/mL TGF-β1 for a further (B) 16 h for TREM-2 mRNA or (C) 48 h for TREM-2 protein expression. mRNA expression was measured by qRT-PCR, values are expressed as either % (A) or fold (B) change relative to the control, normalised to GAPDH ± SEM, *p ≤ 0.05 (one-way ANOVA with post hoc Bonferroni test). TREM-2 protein expression was measured by western blot analysis. β-actin was used as a loading control (n = 3).

PPARγ is also activated by TGF-β1 and the ERK1/2 and p38 MAPK kinase signalling pathways [483, 486, 487]. In addition, analysing the TREM-2 promoter using TRANSFAC® identified two predicted binding regions for PPARγ, suggesting that this transcription factor may regulate TREM-2 expression (Figure 5.10).

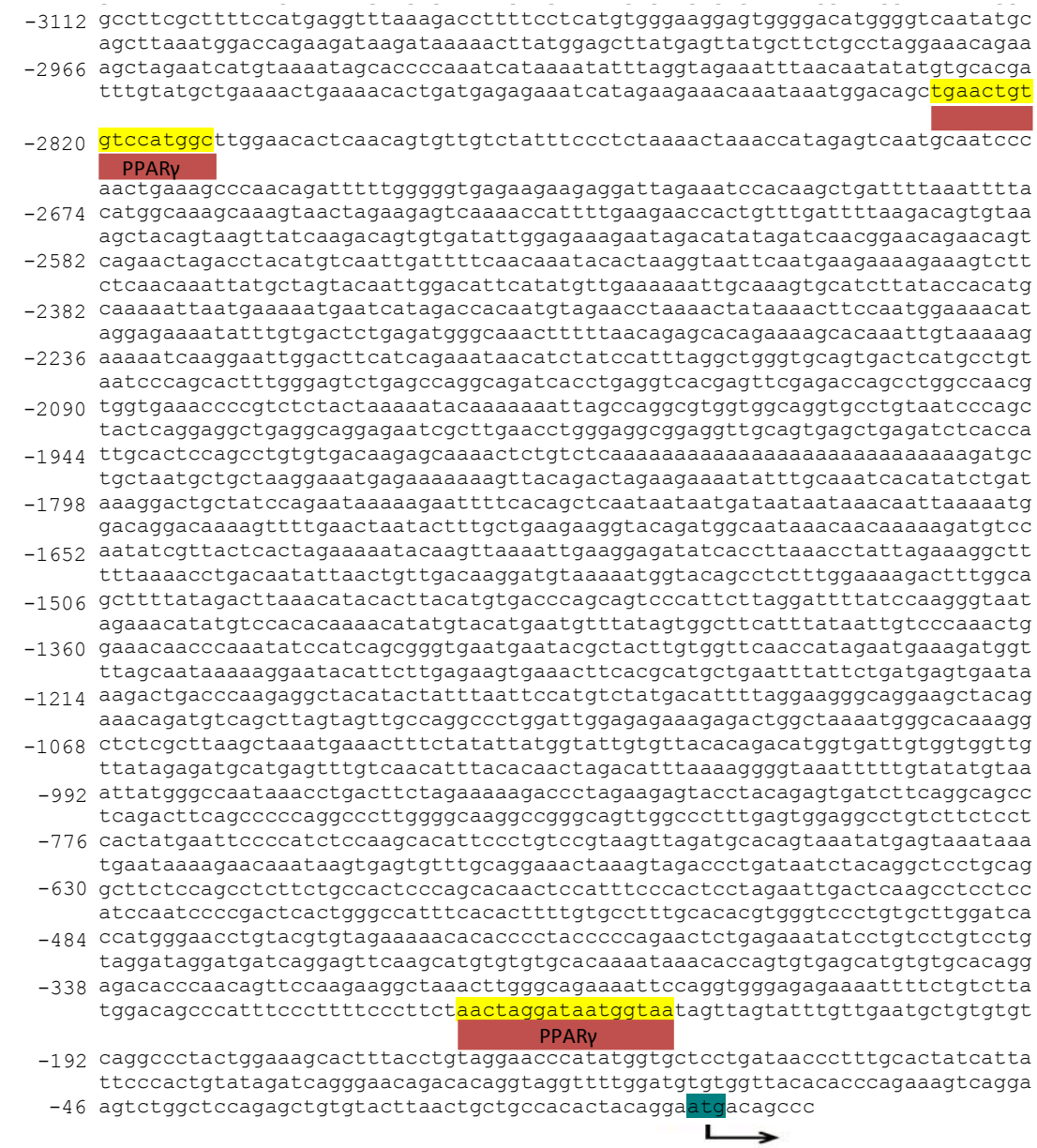

Figure 5.10: Predicted PPARγ binding sites in the TREM-2 promoter. Predicted PPARγ binding sites were identified in the TREM-2 promoter identified by TRANSFAC. The TREM-2 gene start site is indicated by the arrow the bases of the ATG start codon are numbered 0, 1 and 2 respectively.

The effects of GW9662, an inhibitor of PPARγ, on TGF-β1-induced TREM-2 expression were then investigated. GW9662 had no effect on TGF-β1-induced TREM-2 mRNA or protein expression (Figure 5.11A and B), and was confirmed to have no effect on THP-1 cell viability (see Appendix Figure 8.4). This study has eliminated the transcription factors SMAD3, ATF2 and PPARγ from the possible transcription factors required for TGF-β1-induced TREM-2 expression.

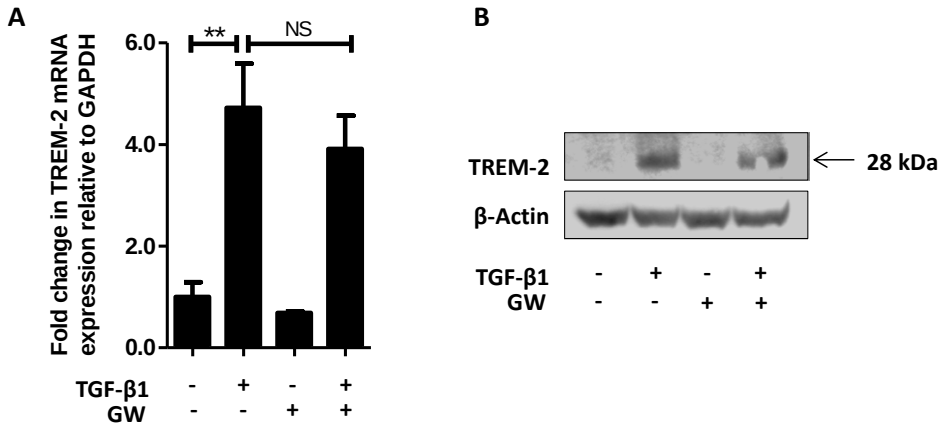

Figure 5.11: Inhibition of PPAY-γ had no effect on TGF-β1-induced TREM-2 expression. THP-1 cells were pre-incubated for 30 min with 10 µM GW9662 (GW) followed by (A) 16 h stimulation with 5 ng/mL TGF-β1 and TREM-2 mRNA measured (n = 3) or (B) 48 h stimulation with 5 ng/mL TGF-β1 and TREM-2 protein measured by western blot analysis (n = 1). TREM-2 mRNA was measured by qRT-PCR, values are expressed as fold change relative to the control, normalised to GAPDH ± SEM, **p ≤ 0.01 (one-way ANOVA with post hoc Bonferroni test), β-actin was used as a loading control.

5.3.5. Effect of TREM-2 KD on TGF-β1 Functions

After observing TGF-β1-induced TREM-2 expression, the roles of TREM-2 in the functions of TGF-β1 were then investigated. In monocytes, TGF-β1 is both pro- and anti- inflammatory and is also thought to be involved in repair and regeneration of tissue [114, 488-490]. TREM-2 siRNA was used to KD TREM-2 expression in THP-1 cells and the effects on TGF-β1-induced cytokine mRNA expression investigated. TREM-2 siRNA inhibited TREM-2 mRNA expression by 73.8% 24 h after transfection (Figure 5.12A). In addition, TREM-2 KD also reduced TGF- β1-induced TREM-2 protein expression (Figure 5.12B).

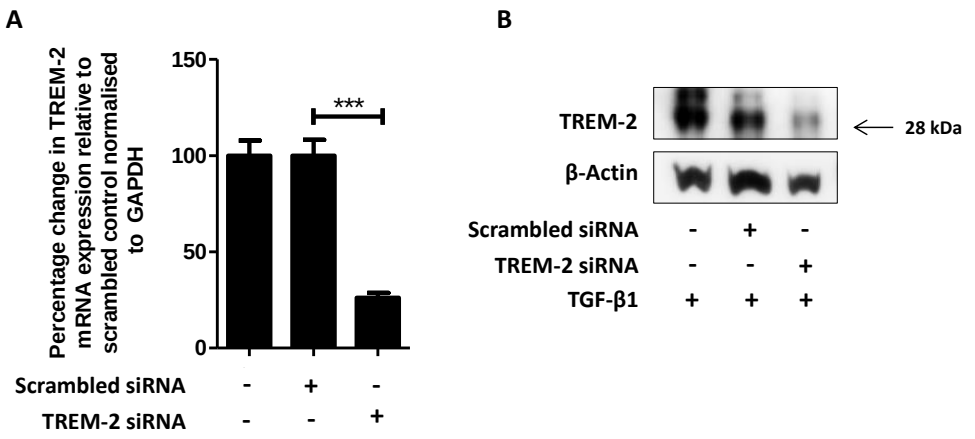

Figure 5.12: TREM-2 siRNA KD in THP-1 cells. THP-1 cells were non-transfected or transfected with 250 nM TREM-2 siRNA or scrambled control for 24 h. (A) TREM-2 expression was analysed by qRT-PCR, values are expressed as % change relative to the control, normalised to GAPDH ± SEM, *** $p \leq 0.001$ (one-way ANOVA with post hoc Bonferroni test). (B) 24 h after transfection, 5 ng/mL TGF-β1 was added for a further 24 h and TREM-2 protein expression measured by western blot analysis, β-actin was used as loading control (n = 3).

In monocytes, TGF- β1 exerts both pro- and anti- inflammatory activities [117, 488], therefore after confirming TREM-2 KD in the THP-1 cells, this study analysed the effect of TREM-2 on TGF-β1-induced proinflammatory gene expression. TGF-β1 caused a moderate increase in the pro-inflammatory cytokines IL-8 and IL-1β ($p \leq 0.001$ and $p \leq 0.05$ respectively) (Figure 5.13A and B). However, TREM-2 KD had no effect on TGF-β1-induced IL-8 and IL-1β expression, suggesting that TREM-2 is not involved in this pro-inflammatory effect of TGF-β1 (Figure 5.13A and B).

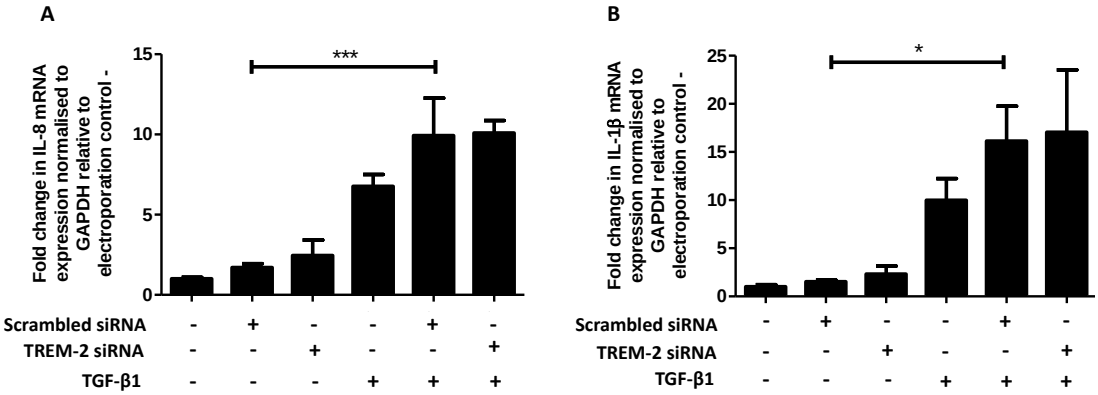

Figure 5.13: TREM-2 is not required for TGF-β1-induced IL-8 or IL-1β in monocytes. THP-1 cells were non-transfected or transfected with 250 nM TREM-2 siRNA or scrambled control, after

24 h, the transfected cells were stimulated with 5 ng/mL TGF-β1 24 h. (A) IL-8 and (B) IL-1β gene expression measured by qRT-PCR, values are expressed as fold change relative to the control, normalised to GAPDH ± SEM, *p ≤ 0.05, ***p ≤ 0.001 (one-way ANOVA with post hoc Bonferroni test) (n = 3).

As well as its effects in inflammation, TGF-β1 is also important for tissue repair and remodelling. Both TREM-2 and TGF-β1 have been shown to be important for efficient wound repair in animal models of wound healing [119, 334]. TGF-β1 mediates tissue repair in part though the regulation of MMPs. MMP-1 is the most characterised MMP for its positive effects on wound healing and repair [33, 491], therefore this study also investigated the effect of TREM-2 KD on TGF-β1-induced MMP-1 expression. TGF-β1 significantly increased MMP-1 mRNA expression in THP-1 monocytes after 24 h stimulation (Figure 5.14A). Interestingly, siRNA KD of TREM-2 significantly reduced TGF-β1-induced MMP-1 by 73.0% suggesting that TREM-2 is required for this change (p ≤ 0.001) (Figure 5.14A).

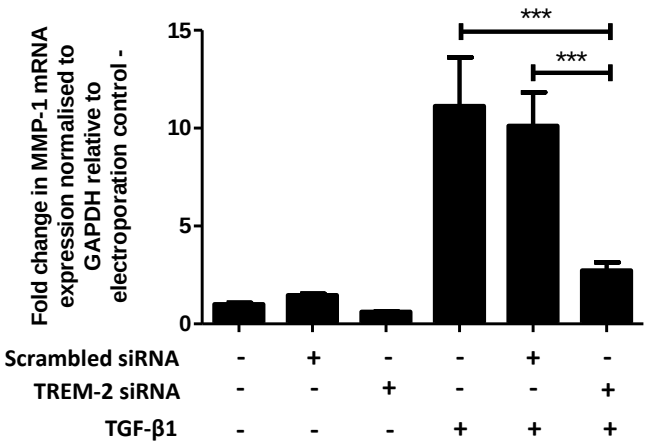

Figure 5.14: TREM-2 is required for TGF-β1-induced MMP-1 expression. THP-1 cells were non-transfected or transfected with 250 nM TREM-2 siRNA or scrambled control. After 24 h, the transfected cells were stimulated with 5 ng/mL TGF-β1 for 24 h. MMP-1 gene expression was measured by qRT-PCR. Values are expressed as fold change relative to the control, normalised to GAPDH ± SEM, *** p ≤ 0.001 (one-way ANOVA with post hoc Bonferroni test) (n = 3).

To confirm that this effect was also found at the protein level, MMP-1 protein expression in THP-1 supernatants were also examined. TGF-β1 increased secreted active MMP-1 protein 48 h after stimulation (Figure 5.15). Active MMP-1 was

detected at 45 and 47 kDa both showing the same pattern of regulation. The 47 kDa band is likely to be the glycosylated form of MMP-1 which has been well documented [492]. This was slightly inhibited in the presence of the control scrambled siRNA, but completely inhibited in the presence of TREM-2 siRNA (Figure 5.15). This study confirms that TREM-2 mediates the increase in active MMP-1 following TGF-β1 stimulation in THP-1 cells.

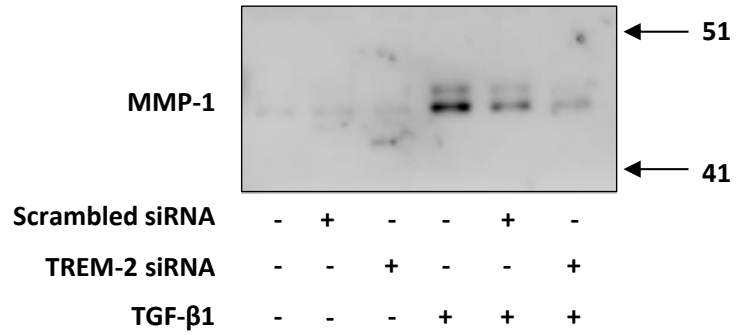

Figure 5.15: TREM-2 is required for TGF-β1-induced secreted MMP-1 protein. THP-1 cells were non-transfected or transfected with 250 nM TREM-2 siRNA or scrambled control. After 24 h, the transfected cells were stimulated with 5 ng/mL for TGF-β1 48 h. MMP-1 protein expression was measured in (A) supernatants by western blot analysis (n = 3).

To analyse the role of TREM-2 and TGF-β1 in MMP-1 regulation and inflammation, the effect of TGF-β1 and TREM-2 KD on LPS-induced MMP-1 expression was examined. MMP-1 was significantly increased up to over 600 fold 24 h after LPS stimulation (Figure 5.16). Co-incubation of LPS and TGF-β1 significantly reduced LPS-induced MMP-1 mRNA expression by 53.0% (Figure 5.16). However, the presence of either the scrambled control or TREM-2 siRNA reduced LPS-induced MMP-1 mRNA similarly to the LPS and TGF-β1 co-stimulation (Figure 5.16) and therefore the role of TREM-2 in this system could not be studied. However, this study does highlight the difference between LPS and TGF-β1-induced MMP-1 mRNA expression.

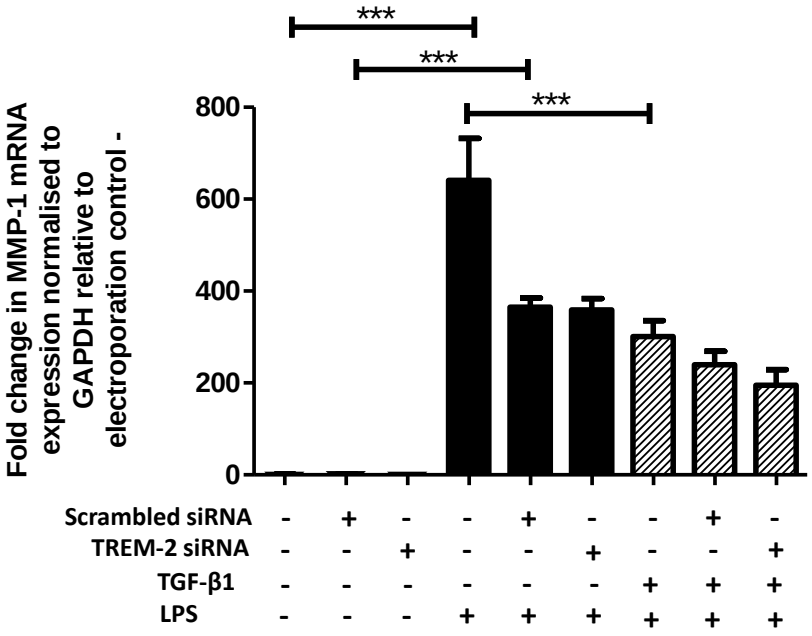

Figure 5.16: TGF-β1 reduces LPS-induced MMP-1 expression. THP-1 cells were non-transfected or transfected with 250 nM TREM-2 siRNA or scrambled control. After 24 h, the transfected cells were stimulated with 10 µg/mL LPS in the presence or absence of 5 ng/mL TGF-β1 for 24 h. MMP-1 gene expression was measured by qRT-PCR, values are expressed as fold change relative to the control, normalised to GAPDH ± SEM, ***p ≤ 0.001 (one-way ANOVA with post hoc Bonferroni test) (n = 3).

5.4. Discussion

TREM-2 expression is increased in inflammatory conditions, including COPD and MS [242, 243]. Similarly to TREM-2, TGF-β1 regulates the inflammatory response and the repair and resolution of tissue following injury and therefore TGF-β1 was investigated for its effects on TREM-2 expression in this study [119, 121, 488]. TGF-β1 was identified as a novel inducer of TREM-2 expression. TGF-β1-induced TREM-2 expression in THP-1 cells was independent of SMAD3 but required the MEK1/ERK1/2, p38 MAP kinase and PI3K signalling pathways. The ERK1/2 MAP kinase signalling pathway was required for TGF-β1-induced TREM-2 protein but not mRNA expression, identifying a post-translational mechanism of TREM-2 regulation by TGF-β1. TGF-β1 activated p38 MAP kinase and PI3K were important for TGF-β1-induced TREM-2 mRNA and protein expression, suggesting that these mediators are upstream of ERK1/2 in the pathway. Furthermore, TREM-2 KD had no effect on TGF-β1-induced IL-1β or IL-8 expression but significantly reduced TGF-β1-induced MMP-1 mRNA and secreted protein expression. Together, these results suggest that TGF-β1 may be in part responsible for the increase in TREM-2 expression observed in inflammatory conditions and in wound healing and that the repair functions of TGF-β1 may require TREM-2.

This study showed that TGF-β1 increased TREM-2 expression in THP-1 cells and primary monocytes and this may be responsible for the increase in TREM-2 seen in inflammatory conditions. To investigate this theory further, future experiments will use TGF-β1 KO mice to investigate the role of this cytokine in TREM-2 upregulation in animal models of disease. Although TGF-β1 plays an important role in immunomodulation, TGF-β1 is also involved differentiation of macrophage dendritic cell progenitors (MDPs) into osteoclasts, dendritic cells or macrophages [123, 125, 126]. TGF-β1 is required for differentiation of MDPs into these cell types, but requires other growth factors including M-CSF for this change, therefore TGF-β1 may be increasing TREM-2 expression in monocytes by inducing differentiation into another cell type. However, although TREM-2 expression remained high up to 7 days in the presence of TGF-β1, removing TGF-β1 after 48 h reduced TREM-2 expression back to basal levels, suggesting that if the increase in TREM-2 expression is due to a change in cell type, it is not due to a permanent change.

In contrast to its effects in monocytes, TGF-β1 did not increase TREM-2 expression in PMA-differentiated THP-1 cells. Perhaps TGF-β1 can only induce TREM-2 expression in macrophages expressing low levels of TREM-2 e.g. M1 type [240]. This

is also supported by the fact that PMA increases TREM-2 expression in THP-1 cells as shown in Section 3.3.2 and TGF-β1 cannot increase TREM-2 expression further than this. Future work will aim to assess the ability of TGF-β1 to induce TREM-2 expression in alveolar and peritoneal macrophages and other primary monocyte-derived macrophages types such as the classical M1-type.

The mechanisms of TREM-2 upregulation are currently unknown. TGF-β1 signals via a variety of pathways including the SMAD, PI3K and MAPK pathways [(133)]. Inhibition of PI3K significantly inhibited TGF-β1-induced TREM-2 mRNA expression. A similar effect was also observed at the protein level, suggesting that PI3K is in part required for TGF-β1-induced TREM-2 expression. However, PI3K inhibition has been previously shown to reduce basal levels of TREM-2 expression and therefore this effect may be in part due to the effect of PI3K inhibition on basal TREM-2 expression [(241)]. Inhibition of the MEK1/ERK1/2 signalling pathway revealed a post-translational mechanism of TREM-2 regulation by TGF-β1, suppressing TGF-β1-induced TREM-2 protein but not mRNA expression. ERK1/2 has been previously been shown to affect mRNA stability following TGF-β1 activation by binding to CU-rich elements in mRNA sequences such as iNOS [(493)] and therefore this may explain how the ERK1/2 signalling pathway regulates TGF-β1-induced TREM-2 expression. Interestingly, inhibition of p38 MAP kinase inhibited both TREM-2 mRNA and protein induced by TGF-β1, suggesting that this kinase is required upstream of TREM-2 mRNA in the pathway. PI3K, ERK and p38 MAP kinases have all been shown to signal together previously following TGF-β1 activation, for example in TGF-β1-induced keratinocyte migration [(494)]. In addition, both PI3K and p38 MAP kinase are required for TGF-β1-induced monocyte migration, confirming the importance of these signalling pathways in monocytes [(488)]. Future studies will investigate if TREM-2 is also required for migration in these cell types.

To further understand how TGF-β1 increases TREM-2 expression, p38 MAP kinase-induced transcription factors were examined. The SMAD pathway is the most characterised signalling pathway activated following TGF-β receptor activation. The SMAD cascade primarily involves SMADs 2 and 3 that dimerise and form a complex with SMAD4, which initiates translocation into the nucleus and binding to SRE in gene promoters [(130)]. In addition, p38 MAP kinase has been shown to increase SMAD3 signalling, which supported the investigation of SMAD3 signalling in TGF-β1-induced TREM-2 expression [(484)]. SMAD3 expression was knocked down to analyse the role of this transcription factor in TGF-β1-induced TREM-2 expression. However, siRNA KD of SMAD3 had no effect on TGF-β1-induced

TREM-2 expression in THP-1 cells, showing that this pathway is SMAD3 independent. However, KO of SMAD3 alone does not eliminate the requirement for the SMAD pathway altogether. Although SMAD3 is required for some of the actions of TGF-β1, in some cases either SMAD2 or 3 can be used [495], therefore to eliminate SMAD signalling, future studies will use a SMAD2/3 double KO to confirm that TGF-β1-induced TREM-2 expression is SMAD independent.

ATF2 is also activated by TGF-β1 and has been shown to be increased by p38 MAP kinase following TGF-β receptor activation [482, 496, 497]. In addition, ATF2 signals with SMAD3 following TGF-β1 activation [498]. However, ATF2 also forms homodimers and heterodimers with other AP-1 family members including Jun, Maf and Fos proteins [499]. The protein that ATF2 dimerises with affects the target response element in promoter regions which include cAMP, stress and ultraviolet response elements, altering transcriptional regulation [498, 500]. However, this study found that ATF2 was not involved in TGF-β1-induced TREM-2 mRNA or protein production. Another transcription factor activated by TGF-β receptor activation is PPARγ [483]. PPARγ is activated by ERK1/2 and p38 MAP kinase (depending on cell type), following TGF-β receptor activation [486, 487]. TGF-β1-induced PPARγ expression has previously been observed in THP-1 cells and has been shown to be responsible for reducing cytotoxic T-cell stimulation and therefore may be responsible for some of the anti-inflammatory effects of this cytokine [483, 501]. In addition, TRANSFAC analysis identified 2 potential PPARγ binding sites on the TREM-2 promoter, suggesting that this transcription factor regulates TREM-2 expression. However, this study shows that this pathway is not involved in TGF-β1-induced TREM-2 expression. This suggests that there is another p38 MAP kinase-induced transcription factor responsible for TREM-2 gene regulation in these cells.

Although this study did not identify the transcription factor(s) required for transcriptional regulation of TREM-2 by TGF-β1, there are still many that could be investigated. AP-2 and the AP-1 transcription factors Jun and c-fos have been shown to be increased following TGF-β1-induced p38 MAP kinase activation and therefore could also be investigated for their role in TREM-2 regulation [502, 503]. In addition, although this study suggests that SMAD3 is not involved, it has been shown that SMAD2 can compensate for SMAD3 in some situations and as previously discussed is important to consider this in our system [504].

The functional role of TGF-β1-induced TREM-2 expression was investigated in this study. If TGF-β1 does in fact increase TREM-2 expression in disease, then perhaps

TREM-2 is required for the effects of TGF-β1 in the tissue repair or the inflammatory response. As previously discussed TGF-β1 has several anti-inflammatory effects. However, in monocytes, TGF-β1 has also been shown to exert some pro-inflammatory effects, including increased monocyte infiltration in inflamed tissues [488, 505]. This study looked at the effect of TREM-2 KD on TGF-β-induced pro-inflammatory cytokine induction. TGF-β1 increased IL-8 and IL-1β expression in THP-1 cells. However, KD of TREM-2 showed that TREM-2 was not required for this process, showing that TREM-2 is not responsible for these pro-inflammatory functions of TGF-β1.

Aside from the immunomodulatory functions of TGF-β1, this cytokine also has many other important properties. TGF-β1 is well recognised for its ability to cause fibrosis and increase tissue remodelling [506, 507]. Although these processes exacerbate some inflammatory conditions such as pulmonary fibrosis, they are caused by the exaggerated repair functions of TGF-β1 [508]. TGF-β1 has been shown to increase epithelial cell migration and angiogenesis, which are both required for wound repair [466, 470]. In addition, TGF-β1-KO mice have inefficient wound repair, further highlighting its importance in this process [119]. The repair functions of TGF-β1 are due to the regulation of extracellular matrix proteins e.g. MMPs [509]. MMP-1 is a well characterised MMP in wound repair. Like TGF-β1, MMP-1 is essential for efficient wound healing [33, 491]. However, the effect of TGF-β1 on MMP-1 expression in monocytes has not been published previously and therefore this study measured the effect of TGF-β1 on MMP-1 expression in the THP-1 monocytic cell line. Interestingly, MMP-1 mRNA expression and protein secretion were increased by TGF-β1 stimulation. TGF-β1 has been shown previously to regulate MMP-1 in fibroblasts, but in contrast to the effects observed here in THP-1 cells, TGF-β1 has an inhibitory effect [510-512]. Since both TGF-β1 and MMP-1 have important functions in tissue repair and remodelling, it is likely that in THP-1 cells, TGF-β1-induced MMP-1 secretion is important for these functions of TGF-β1. To investigate the role of TREM-2 in this process, THP-1 cells were transfected with TREM-2 siRNA to KD gene expression. TGF-β1-induced MMP-1 mRNA expression and protein secretion was significantly inhibited with TREM-2 KD, suggesting that TREM-2 is required for TGF-β1-induced MMP-1 expression.

TGF-β1 has demonstrated both pro- and anti-inflammatory properties in monocytes. To understand which role TGF-β1 is playing in this system, this study analysed the effect of TGF-β1 on LPS-induced MMP-1 and compared TGF-β1-induced MMP-1 to that of LPS. MMP-1 mRNA expression was significantly higher with LPS than with

TGF-β1 alone. In addition, co-stimulation of TGF-β1 and LPS significantly reduced LPS-induced MMP-1 expression, suggesting that TGF-β1 is exerting an anti-inflammatory effect in this system, further suggesting that TGF-β1 increases MMP-1 expression for its repair functions. Unfortunately, the role of TREM-2 in this process could not be studied, because adding either TREM-2 or scrambled control siRNA, suppressed LPS-induced MMP-1 levels to that of LPS and TGF-β1 co-stimulation, which may be due to the effect of the siRNA on TLR activation. Despite this, this study suggests that in pro-inflammatory environments, TGF-β1 reduces MMP-1 expression, but in non-inflammatory conditions, increases MMP-1 it due to its requirement for the repair and resolution functions of TGF-β1. Studies have shown that both TREM-2 and MMP-1 are important for efficient wound healing [119, 491]. MMP-1 and TGF-β1 increase wound healing by regulating the extracellular matrix to increase angiogenesis and epithelial migration, but these two proteins have not been previously linked for these functions [34, 513-515]. This study suggests that the repair functions of TGF-β1 may be in part through TREM-2-induced MMP-1 secretion.

To continue this project, future work will analyse the role of SMAD2 and 4 in TGF-β1-induced TREM-2 expression in THP-1 cells. After ruling out these transcription factors, this study will analyse the TREM-2 promoter sequence to identify other transcription factors that may be required for this process. Another aim is to understand more about the functional roles of TGF-β1-induced TREM-2 expression. Future studies will aim to confirm the evidence shown here that TREM-2 is not exerting an inflammatory effect, by analysing the effect of TGF-β1 on the secretion of other cytokines in THP-1 cells. Future studies will also investigate the effect of TREM-2 KD on TGF-β1-induced MMPs and their regulators including TIMPs and the relationship between TGF-β1, MMP-1 and TREM-2 in relation to wound repair. Monocyte-epithelium co-culture models and scratch assays will be used to measure the effects of TREM-2 in TGF-β1 and MMP-1-induced epithelial cell migration. In addition, 3D culture models and gene expression of angiogenic factors will be used to measure the effects of TREM-2 in TGF-β1 and MMP-1-induced angiogenesis. If TGF-β1 is in fact signalling via TREM-2 and MMP-1 to improve epithelial migration and angiogenesis *in vitro,* the next stage would be to test this hypothesis *in vivo*.

5.5. Summary

In conclusion, this study revealed that TGF-β1 increased TREM-2 expression in THP-1 monocytes though a p38/ERK1/2 MAP kinase-dependent signalling pathway, suggesting that TGF-β1 may in part be responsible for the increase in TREM-2 expression observed in inflammatory conditions. In addition, this study showed a novel activity of TREM-2 in the regulation of TGF-β1-induced MMP-1. Due to the role of MMP-1, TGF-β1 and TREM-2 in wound repair, this study suggests that TGF-β1 improves wound repair through MMP-1 by a mechanism that requires TREM-2.

6. Investigating TREM-2 Expression in Ischemia Using an *In Vitro* Oxygen Glucose Deprivation Co-Culture Model

Acknowledgments:

Megan Drew, an MPharm undergraduate project student, helped produce the data for Figure 6.8.

6.1. Introduction

TREM-2 is increased in inflammatory conditions including MS where it is thought to play a role in the resolution of inflammation and injury [243]. Through a collaboration with Prof. Otto Witte and Dr. Christiane Frahm from the University of Jena, Germany, our laboratory has previously investigated gene expression changes in the brain following stroke, which suggested that TREM-2 may also be involved in the resolution of inflammation in stroke. In this experiment, C57Bl6 mice were sham operated or had the middle cerebral artery occluded for 30 min and then the vessel released to allow reperfusion of blood back into the brain. Ipsilateral and contralateral brain hemispheres were collected 2 and 7 days after reperfusion (Figure 6.1) and microarray analysis performed to identify any changes in inflammatory gene expression. TREM-2 was identified as one of the most highly upregulated genes (6-fold increase over both controls) 7 days after reperfusion. At this time, the brain begins to resolve the inflammation and repair the damage caused by occlusion, thereby identifying TREM-2 as a possible mediator during the resolution phase following stroke [516]. Since TREM-2 has important roles in dampening down inflammation and in phagocytosis, the increase in TREM-2 in this model suggests that these functions of TREM-2 may be required for repair of the brain following stroke.

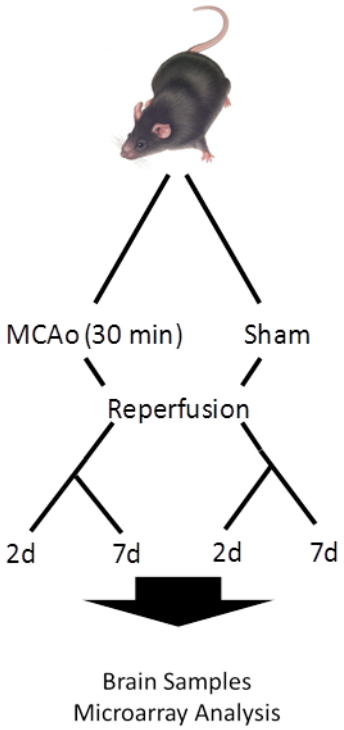

Figure 6.1: MCAo mouse model of stroke. Mice were either sham operated (Sham) or had their MCA occluded for 30 min. Ipsilateral and contralateral brain hemispheres were collected for microarray analysis 2 and 7 days after reperfusion.

TREM-2 is mainly expressed in microglia in the brain and therefore it is likely that the functions of TREM-2 are mediated by these cells. Microglia are the immune cells of the brain that become activated in inflammation and during ischaemia, by changes in the surrounding environment, including inflammatory mediators and high levels of glutamate [(517)]. Microglia are closely associated with neurons. In healthy conditions, neurons send signals to microglia to suppress their activation, for example expression of CD200 on healthy neurons is detected by the CD200 receptor on microglia, and this ligand receptor interaction results in suppression of microglial activation [(518-520)]. When neurons become damaged or stressed, for example in ischaemia, the neurons stop sending these signals and produce other signals e.g. ATP and UDP that bind to receptors on the surface of microglia to induce microglial activation [(521-523)].

Chapters 4 and 5 have shown that IL-4 and TGF-β1 increase TREM-2 expression in monocytes. However, the reason for the increase in TREM-2 expression in stroke is unknown. Identifying the cause of this increase and the cell types involved is important to understand how TREM-2 is regulated, which will be useful information

when considering TREM-2 as a therapeutic target. To study further the increase in TREM-2 expression observed in cerebral ischaemia, this study established an *in vitro* model of ischaemia to investigate the changes in TREM-2 expression observed in the *in vivo* model of stroke.

6.2. **Aims**

The aims of this study were to:

- Establish an *in vitro* model of ischaemia reperfusion.
- Use this model to study the increased TREM-2 expression seen *in vivo* with ischaemia reperfusion and determine the mechanisms involved.

6.3. Methods

6.3.1. Cell Culture

The N9 murine microglial cell line was kindly donated by Dr. Ji Ming Wang at The Center for Cancer Research, National Cancer Institute at Frederick (US) [(394)]. The cells were grown in IMDM media (PAA) with 4.5 g/L glucose, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 10% FCS. For OGD experimentation, N9 cells were maintained in DMEM with or without glucose and with 1% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM L-glutamine. The cells were passaged one in ten using a cell scraper every 3-5 days. N9 cells were used up to passage 20.

SH-SY5Y cells are a human neuroblastoma cell line and were kindly donated by Dr. Marcus Rattray, The University of Reading (UK) [(395)]. The cells were grown in DMEM media containing 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 4.5 g/L glucose. For OGD experimentation, SH-SY5Y cells were maintained in DMEM with or without glucose and with 10% or 1% FCS as indicated. The cells were passaged one in ten every 3 days, the suspension cells were centrifuged at 1200 rpm for 5 min and adherent cells removed using 0.25% trypsin/EDTA ensuring both suspension and adherent cells were maintained. In some experiments, SH-SY5Y cells were differentiated with 10 µM all-trans retinoic acid (Sigma) for 9 days, the media was replaced and all-trans retinoic acid removed 24 h before experimentation. Cells were used up to passage 20 [(524)].

6.3.2. OGD

N9 and SH-SY5Y cells were seeded into 6-well plates until they reached 80% confluency (1 x 10^6 cells/well). The media was then changed to either control or glucose free DMEM (PAA). For OGD conditions, the cells were placed into the modular incubator chamber (Billups-Rothenberg Inc) (Wolf Laboratories Limited, York, UK) and flushed through with N_2-containing 5% CO_2 for 10 min to remove any remaining oxygen and the chamber sealed. Control cells were left at RT whilst the oxygen was removed from the OGD cells, then the control and OGD cells were placed back into the 37°C incubator for 30 min or 2 h (N9 cells) or 24 h (SH-SY5Y cells). For the N9 OGD experiments the cells were reperfused for up to 5 days, for this, the cells were removed from the oxygen-free modular incubator chamber and

the media was changed to DMEM (1% FCS) with glucose and left in culture for up to 5 days and a media change every other day.

6.3.3. Co-culture Experiments

N9 cells were cultured alone in IMDM containing 10% FCS or with control (non-OGD) SH-SH5Y cells or SH-SY5Y cells 24 h after OGD (Figure 6.2). SH-SY5Y cells and supernatant, cells only or supernatant only were added to N9 cells for 4 h (Figure 6.2). Samples were also included to control for the SH-SY5Y supernatant samples, by adding DMEM media with or without glucose to the N9 cells (in IMDM). The SH-SY5Y cells were removed from cell culture plates using a cell scraper and either added directly in suspension to N9 cells or centrifuged at 300 x g for 5 min and the supernatant or cell pellet added separately. In all cases where media was added, 900 µL of media was removed from the N9 cells and replaced with 900 µL of SH-SY5Y supernatant, cells with supernatant or DMEM media. Where SH-SY5Y cell pellets were added, the cell pellet from one well of SH-SY5Y cells (~one million cells) was resuspended in 900 µL of N9 media from the corresponding well and added back into the well containing the N9 cells (Figure 6.2). To collect the protein samples, the N9 cells were washed with PBS to remove the SH-SY5Y cells and the samples lysed using 1X SDS sample buffer as described (Section 8.1.3). Protein samples were analysed for TREM-2 expression using western blot analysis as described (Section 2.5).

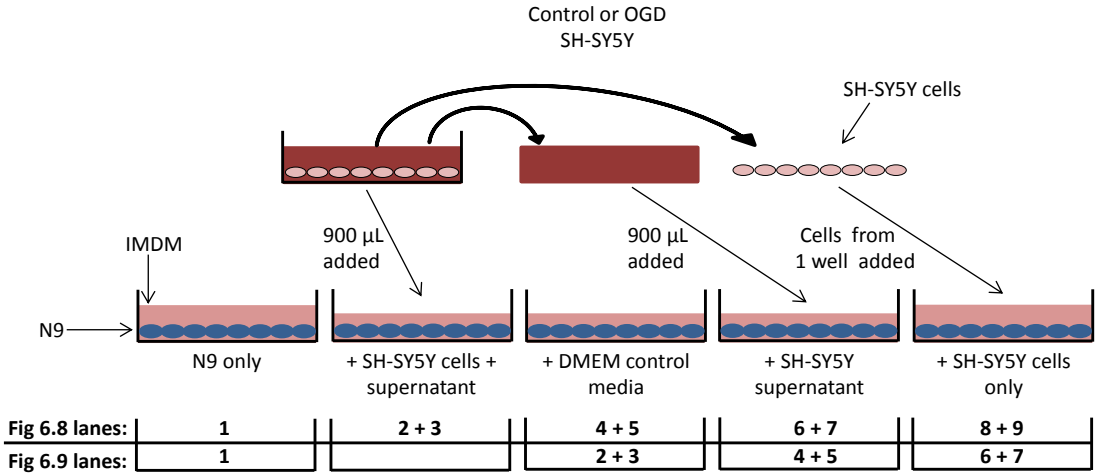

Figure 6.2: Method for analysing the effect of adding control or OGD SH-SY5Y cells on TREM-2 expression in N9 microglial cells. After 24 h in control or OGD conditions, the SH-SY5Y cells were removed using a cell scraper. 900 µL of SH-SY5Y cells plus supernatant were added to the corresponding N9 well. 900 µL of control or glucose free DMEM was added to the N9 cells to control for the addition of the media to the N9 cells. SH-SY5Y cells and supernatants were separated and 900 µL of supernatant added to the corresponding N9 cell. The SH-SY5Y cell pellets were resuspended in the IMDM media from the corresponding N9 well and added into the N9 well.

To optimise the change in TREM-2 expression observed in N9 cells following the addition of control SH-SY5Y cells, different amounts of SH-SY5Y cell pellets were added to N9 cells from 1-4 x 10^6 cells (1-4 wells of a 6 well plate) for 4 h. After this time the cells were lysed as described above and cell lysates measured for TREM-2 expression by western blot analysis as described in (Section 2.5) or by immunofluorescence. For immunofluorescence, the SH-SY5Y cells were stained with 10 µM carboxyfluorescein succinimidyl ester (CFSE) (AAT Bioquest, CA, US) for 10 min at 37°C followed by three washes in warmed PBS by centrifugation at 300 x g for 10 min. After the washing, they were added to the N9 cells as above. Immunofluorescence was performed as previously described (Section 2.6.1).

6.3.4. Measurement of SH-SY5Y Cell Death

The cell viability of SH-SY5Y (1 x 10^5 cells undifferentiated or 1.4 x 10^5 cells differentiated) was measured by MTS assay following 24 h OGD or control conditions (as described in section 2.3). Each run was performed in triplicate.

SH-SY5Y cell apoptosis was measured by Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. For these experiments, SH-SY5Y cells were seeded at 1 x 10^6 cells/well onto ethanol-sterilised, poly-L-lysine coated coverslips 16-24 h before experimentation. Following cell treatment (24h OGD or non-OGD) SH-SY5Y cell apoptosis was measured using TUNEL. The DeadEnd™ Fluorometric TUNEL Assay Kit (Promega) measures DNA damage produced in apoptotic cells by binding and fluorescently labelling fragmented DNA. The cells were fixed and permeabilized as described in the immunofluorescence protocol (Section 2.6.1) then incubated at room temperature in equilibration buffer for 5-10 min. The excess equilibration buffer was removed and replaced with 50 µL of terminal deoxynucleotidyl transferase recombinant enzyme (rTdT) incubation buffer (1 µL rTdT enzyme, 5 µL nucleotide mix and 45 µL equilibration buffer) for 1 h at 37°C. The reaction was stopped by adding 1 mL of the SSC solution (NaCl, sodium citrate solution) for 15 min. The slides were washed in PBS two times for 10 min each and then mounted as stated in the immunofluorescence protocol previously described (Section 2.6.1).

Cell culture media was removed from the adherent cells and Trypan Blue (diluted one in ten in PBS) was added and left for one minute to allow staining of the dead cells. The Trypan Blue was washed off with PBS and 2 mL of PBS added to the wells and the cells viewed by light microscopy.

6.4. Results

6.4.1. The Effect of OGD on TREM-2 Expression in the Murine N9 Microglial Cell Line

TREM-2 is expressed in the N9 microglial cell line but not in the SH-SY5Y neuroblastoma cell line (see Chapter 3 Figure 3.8). The high expression of TREM-2 in microglial cells but little to no expression in other CNS cell types has also been shown by other groups and therefore any changes in TREM-2 expression observed in cerebral ischaemia are most likely to be due to changes in TREM-2 expression in microglial cells [81, 149, 258]. This study used OGD to mimic ischaemic conditions in the brain following stroke. Preliminary data confirms the effectiveness of the ischaemia chamber showing that IL-1β and TGF-β1 mRNA are increased during the reperfusion phase following hypoxia which is also seen in the literature (see Appendix Figure 8.5) [525, 526]. The effectiveness of the ischaemia chamber was assessed by aThe process of reperfusion was mimicked by the re-introduction of oxygen and glucose into the media. The effect of OGD on TREM-2 expression in N9 microglial cells was analysed by western blot analysis. N9 cells were in a control environment (in the presence of oxygen and media containing glucose) or exposed to OGD for 30 min or 2 h and oxygen and glucose re-introduced (simulating reperfusion) for up to 5 days. Figure 6.3 demonstrates that OGD had no effect on TREM-2 expression in N9 cells at any of the time points tested. Any small changes observed were not consistent between the replicates, suggesting that the increase in TREM-2 expression in the MCAo model is not due to the direct effect of ischaemia on microglial cells alone.

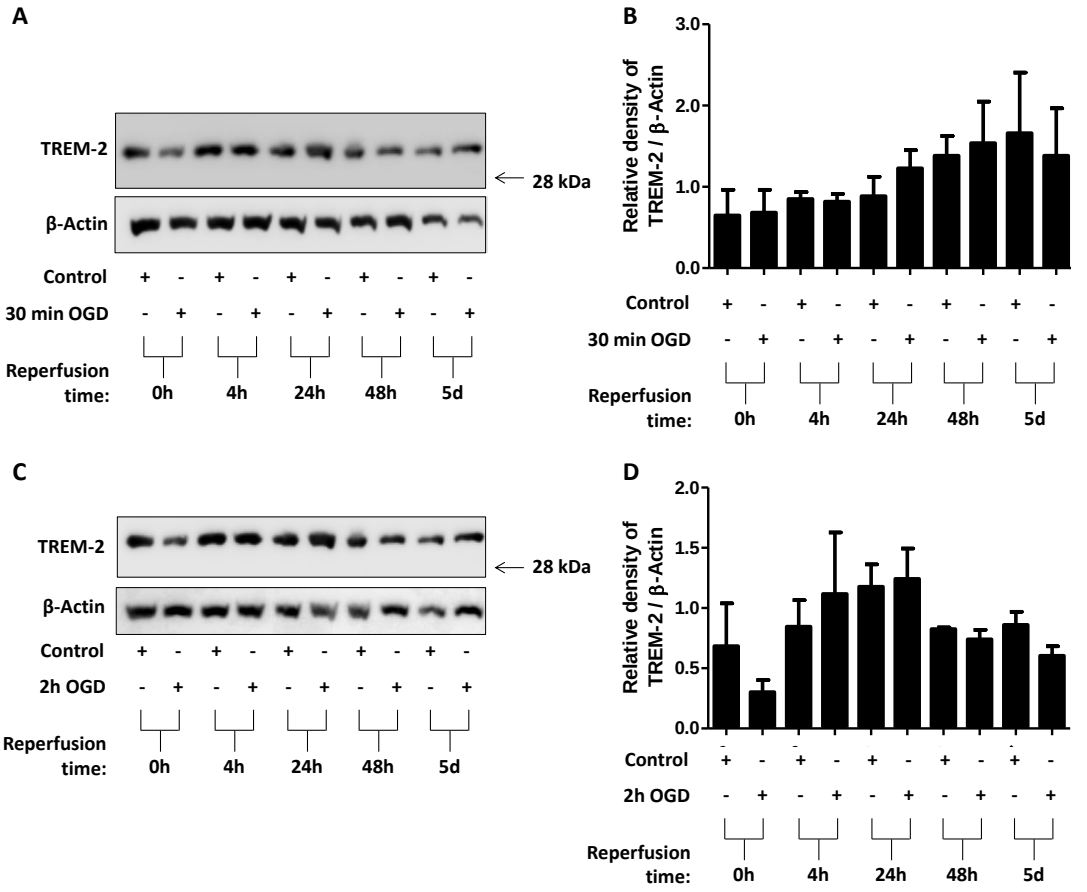

Figure 6.3: OGD does not affect TREM-2 protein expression in N9 murine microglial cells. N9 microglial cells were subjected to either (A and B) 30 min or (C and D) 2 h OGD or control conditions followed by reperfusion (4 h, 24 h, 48 h or 5 days) or no-reperfusion (0 h). TREM-2 expression was measured by western blot analysis. β-actin was used as a loading control. (B and D) Densitometry values of TREM-2 protein expression relative to β-actin. Values are expressed as mean ± SEM (n = 3).

6.4.2. OGD-Induced Cell Death in Differentiated and Undifferentiated SH-SY5Y Cells

The ligand for TREM-2 is present on the surface of apoptotic neurons and binding of the ligand to TREM-2 on microglia leads to phagocytosis [(149)]. The clearance of neurons by TREM-2 may be very important to repair the brain following stroke and minimise inflammation and further damage and therefore, the next stage of this study was to determine if neurons were required for ischaemia-induced TREM-2 expression. SH-SY5Y cells are a cell line frequently used as a neuronal model since they possess many characteristics of primary neurons [(527)]. SH-SY5Y were left un-differentiated or differentiated with 10 µM all-trans retinoic acid for 9 days [(528)]. OGD

was used to simulate ischaemia. The effects of 24 h OGD on undifferentiated and differentiated SH-SY5Y cell death were examined. In undifferentiated SH-SY5Y cells, cell viability was reduced by 82.4% following 24 h OGD, suggesting that OGD increases cell death in this cell type (Figure 6.4).

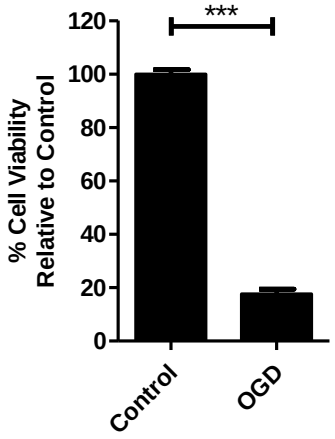

Figure 6.4: OGD reduces undifferentiated SH-SY5Y cell viability. SH-SY5Y cells were cultured alone or OGD conditions for 24 h and cell viability measured by MTS assay. Values are expressed as a percentage of the control absorbance. Mean ± SEM, ***p ≤ 0.001 (unpaired Student's t test) (n = 3).

TUNEL assay was then performed to analyse the type of cell death that occurred following 24 h OGD. TUNEL binds to fragmented DNA present in apoptotic cells. In control conditions, 14.0% of undifferentiated SH-SY5Y cells were TUNEL positive, whereas following OGD, 76.7% of cells were TUNEL positive (Figure 6.5). This suggests that OGD increases apoptosis in undifferentiated SH-SY5Y cells.

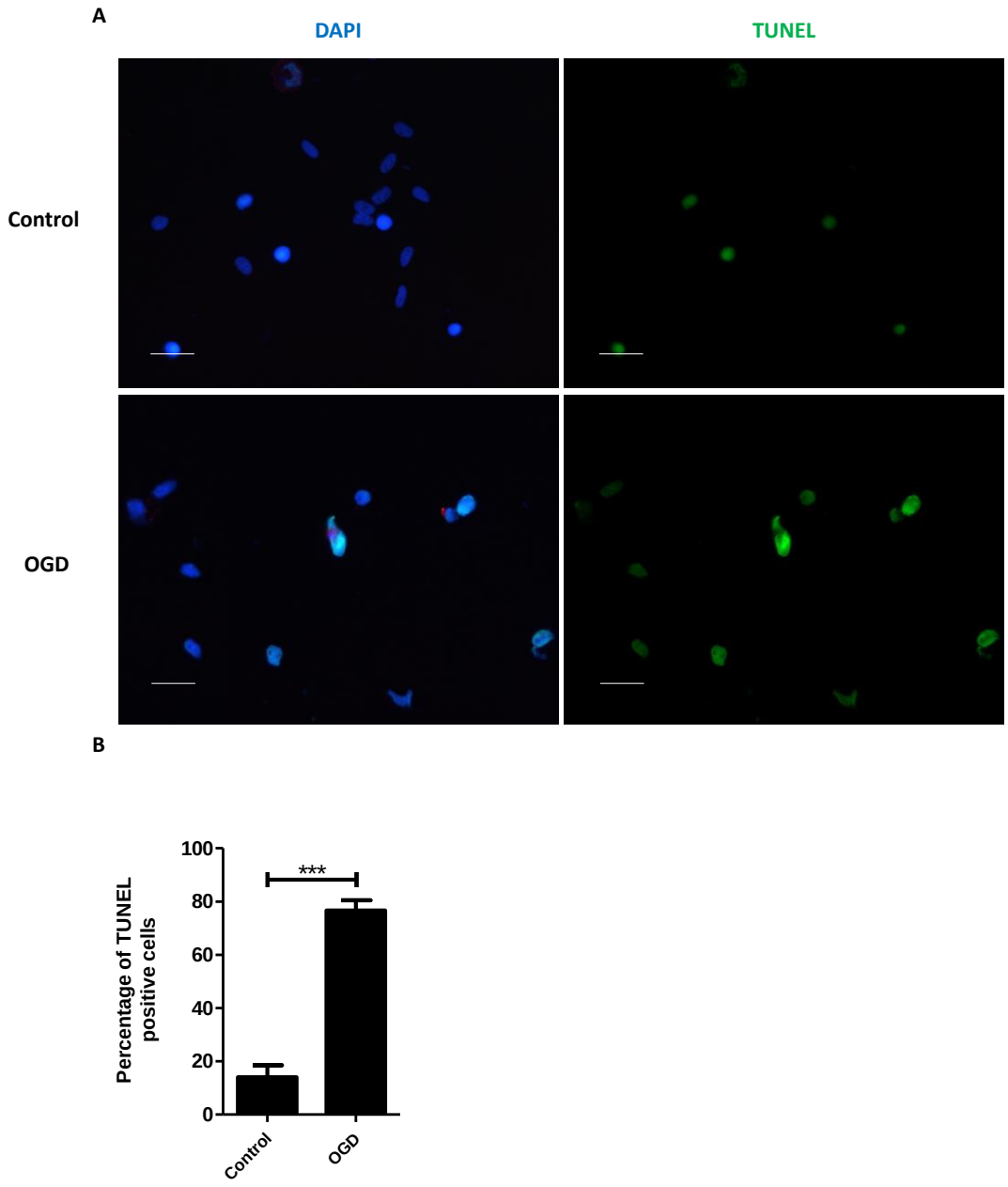

Figure 6.5: TUNEL staining in undifferentiated SH-SY5Y cells after 24 h OGD. SH-SY5Y cell apoptosis was measured after 24 h OGD. Immunofluorescence measured TUNEL staining (GREEN) and the nuclear stain DAPI (BLUE) which allowed identification of TUNEL negative and positive cells. (A) Immunofluorescence image of TUNEL and DAPI labelling in SH-SY5Y cells. (B) The percentage of TUNEL positive cells were measured by counting the number of TUNEL positive (GREEN) cells in 100 cells, ***p ≤ 0.001 (unpaired Student's t test) (n = 3). Scale Bar 50 µm.

After observing the effect of OGD on SH-SY5Y neuroblastoma cells, they were differentiated into a more neuronal-like cell with 10 µM all-trans retinoic acid for 9

days. Differentiation was confirmed by observation of altered morphology including elongated neurites and by suppression of proliferation [529]. The effects of OGD-induced apoptosis were compared to that of undifferentiated cells. OGD reduced differentiated SH-SY5Y cell viability by 76.8% ($p \leq 0.001$), similarly to the undifferentiated cells (Figure 6.6).

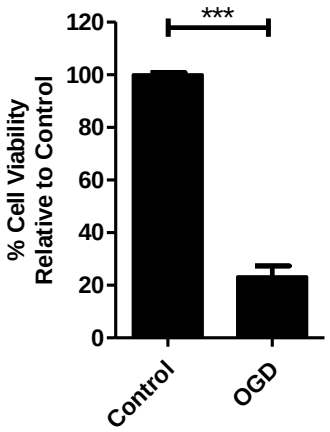

Figure 6.6: OGD reduces cell viability in differentiated SH-SY5Y cells. Cells were differentiated for 9 days with 10 µM all-trans retinoic acid. They were then cultured alone or in OGD conditions for 24 h and cell viability measured by MTS assay. Values are expressed as a percentage of the control absorbance. Mean ± SEM, ***$p \leq 0.001$ (unpaired Student's t test) (n = 3).

To further assess SH-SY5Y cell viability, the differentiated cells were also stained for TUNEL 24 h after OGD. The basal numbers of TUNEL-positive cells were lower in differentiated SH-SY5Y cells (3.7%) compared to undifferentiated cells (14.0%) (Figure 6.7). In addition, in differentiated cells, OGD had no significant effect on TUNEL staining, suggesting that there are inconsistencies between the TUNEL and MTS assays in this experiment. This is likely to be due to the different mechanisms of assessing cell death in these assays. However, TUNEL analysis suggests that OGD induces significant apoptosis in undifferentiated cells but not in differentiated cells (Figure 6.7).

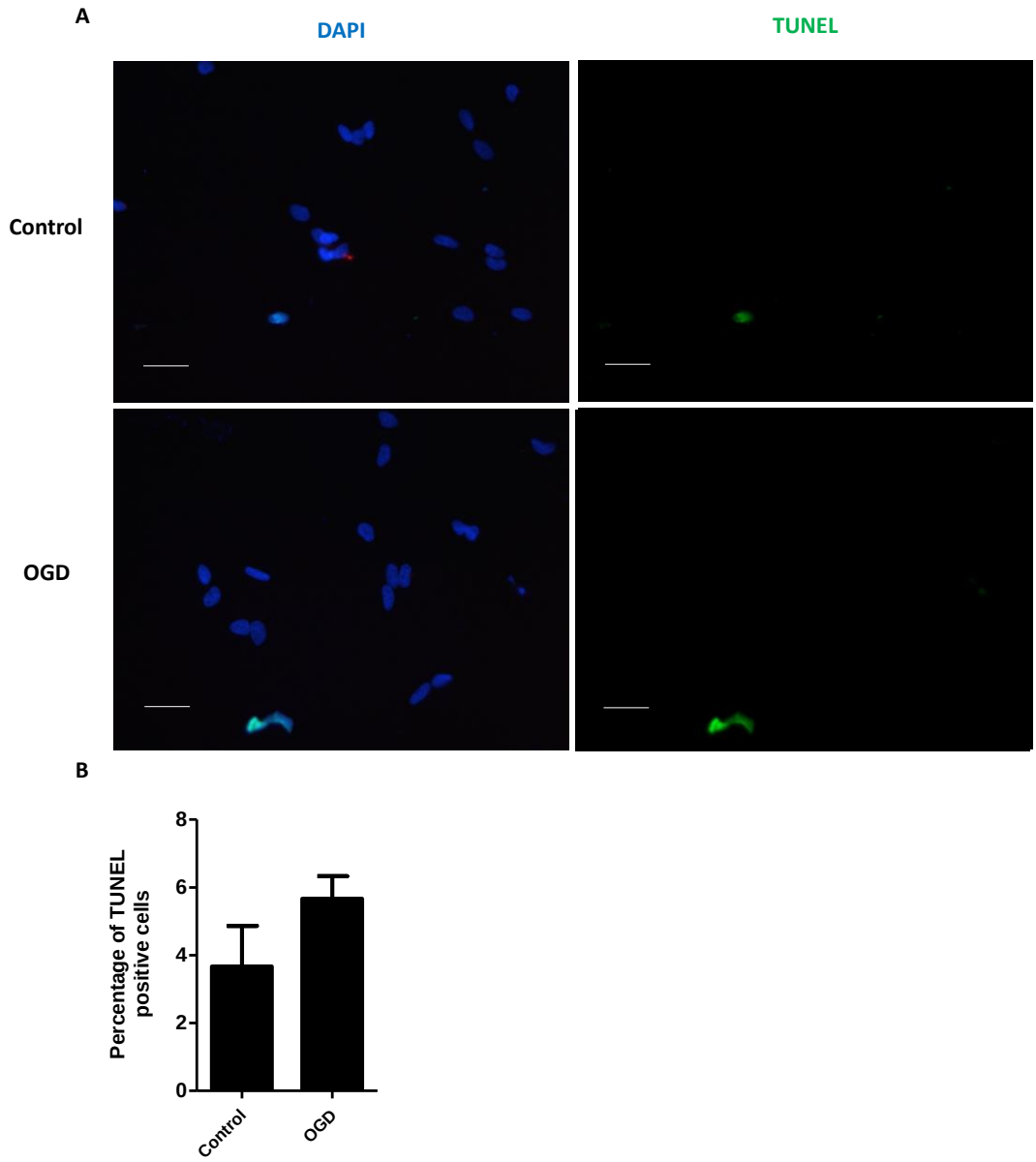

Figure 6.7: TUNEL staining in differentiated SH-SY5Y cells after 24 h OGD. Apoptosis of SH-SY5Y cells differentiated for 9 days with 10 µM all-trans retinoic acid was measured 24 h after OGD. Immunofluorescence measured TUNEL staining (GREEN) and the nuclear stain DAPI (BLUE) which allowed identification of TUNEL negative and positive cells. (A) Immunofluorescence image of TUNEL and DAPI labelling in SH-SY5Y cells. (B) The percentage of TUNEL positive cells were measured by counting the number of TUNEL positive (GREEN) cells in 100 cells. ***p ≤ 0.001 (unpaired Student's t test) (n = 3). Scale Bar = 50 µm.

6.4.3. SH-SY5Y and N9 Co-Culture Following SH-SY5Y Cell OGD

To observe the effects of OGD-treated SH-SY5Y cells on TREM-2 expression in microglia, a co-culture system was designed (Figure 6.2). Undifferentiated SH-SY5Y cells were exposed to 24 h OGD and then co-incubated with N9 cells and TREM-2 measured by western blot analysis. Lane 1 contains N9 cells alone, lanes 2 and 3 are co-incubated with control or OGD SH-SY5Y cells and supernatant which showed a small reduction with co-culture with the control SH-SY5Y cells and supernatant (Figure 6.8 lane 2 compared to lane 1). N9 co-culture with control or OGD undifferentiated SH-SY5Y cell supernatants had no effect on TREM-2 expression compared to N9 adding control or glucose free DMEM media (Figure 6.8 lanes 6 and 7 compared to lanes 4 and 5). However, co-culture of N9 cells with control undifferentiated SH-SY5Y cells only (without the media) caused a modest reduction in TREM-2 expression compared to N9 cells alone (Figure 6.8 lane 8 compared to lane 1). There was also a trend showing a small increase in TREM-2 expression with the addition of OGD SH-SY5Y cells compared to adding control SH-SY5Y cells (Figure 6.8 lane 9 compared to lane 8). This suggests that the presence of non-ischaemic SH-SY5Y cells reduces TREM-2 expression in N9 cells.

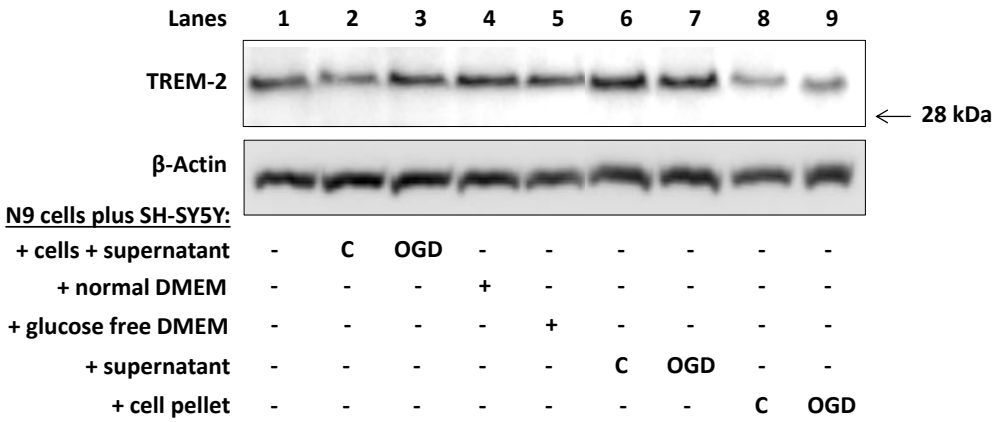

Figure 6.8: Effect of OGD SH-SH5Y cells on TREM-2 expression in N9 cells. N9 cells were cultured for 4 h, alone or in the presence of control (C) or 24 h OGD SH-SY5Y cells + supernatants. N9 cells were also cultured in the presence of control or OGD SH-SY5Y cell supernatants or cell pellets only (1 million cells per sample). To account for the different media required for N9 and SH-SY5Y cell cultures, SH-SY5Y control or glucose free media were added to N9 cells to ensure this alone did not affect TREM-2 expression. TREM-2 protein expression was measured by western blot analysis, with β-actin used as a loading control (n = 3).

To see if the differentiation state of SH-SY5Y cells affected TREM-2 regulation in this model, SH-SY5Y cells, differentiated with all-trans retinoic acid, were co-cultured with N9 cells following 24 h OGD or control conditions. Similarly to undifferentiated SH-SY5Y cells, addition of control cells caused a small but consistent reduction in TREM-2 expression that was not observed with the addition of OGD cells (Figure 6.9 lane 6 and 7). In addition, differentiated SH-SY5Y cell supernatants (lanes 4 and 5) had no effect on TREM-2 expression (Figure 6.9). These results suggest that this effect is independent of differentiation status.

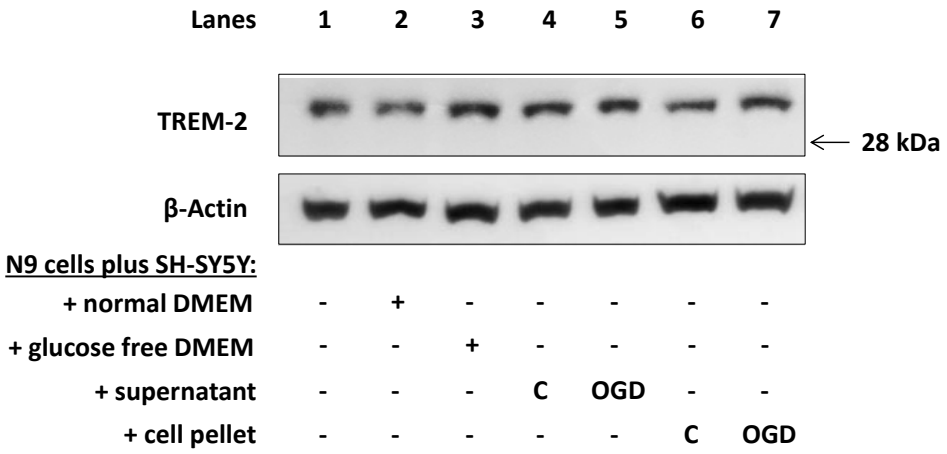

Figure 6.9: The effect of differentiated OGD SH-SH5Y cells on TREM-2 expression in N9 cells. N9 cells were cultured for 4 h, alone or in the presence of control (C) or 24 h OGD SH-SY5Y cells + supernatants differentiated for 9 days with 10 µM all-trans retinoic acid. N9 cells were also cultured in the presence of control or OGD differentiated SH-SY5Y cell supernatants or cell pellets only (1 million cells per sample). To account for the different media required for N9 and SH-SY5Y cell cultures, SH-SY5Y control or glucose free media was added to control N9 cells. TREM-2 protein expression was measured by western blot analysis, β-actin was used as a loading control (n = 3).

To confirm that the addition of SH-SY5Y cells resulted in a reduction in microglial TREM-2 expression, increasing numbers of undifferentiated SH-SY5Y cells co-incubated with N9 cells. Increasing the number of undifferentiated SH-SY5Y cells reduced TREM-2 expression in N9 cells further up to a 3:1 ratio (three million neurons: one million microglial cells) (Figure 6.10). To confirm that this effect did not cause cell death of N9 cells, cell viability was analysed using Trypan Blue exclusion (as described in Section 6.3.4). The increase in cell number had no effect of N9 cell

viability (see Appendix Figure 8.6) therefore three million cells were chosen for future studies.

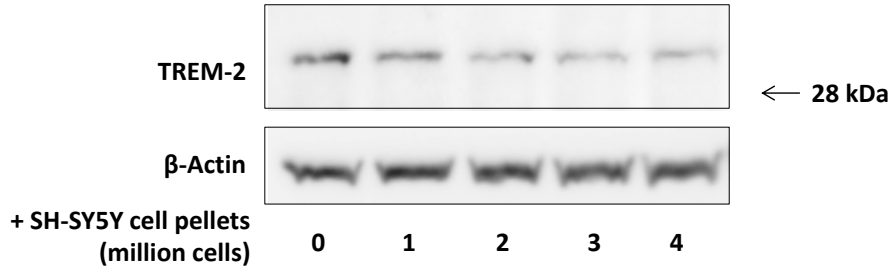

Figure 6.10: Increasing numbers of SH-SH5Y cells reduces TREM-2 expression in N9 cells. N9 cells were cultured for 4 h, alone (0) or in the presence of 1, 2, 3 or 4 million healthy (control) undifferentiated SH-SY5Y cells. After removal of SH-SY5Y cells, N9 cells were analysed by western blot analysis for TREM-2 protein expression. β-actin was used as loading control (n = 3).

Since the increased ratio of undifferentiated neurons: microglial cells resulted in a clear reduction in microglial TREM-2 expression, the effect of increasing control or OGD neurons in the model was investigated. A 3:1 ratio of cells was used, with three million differentiated neurons co-incubated with one million microglial cells and compared with undifferentiated cells. To control for the effect of reduced proliferation in OGD conditions, the SH-SY5Y cells were counted after the experiment and equal numbers of control or OGD cells were co-incubated with N9 cells. The addition of control differentiated SH-SY5Y cells reduced TREM-2 expression in N9 cells more than undifferentiated SH-SY5Y cells after 4 h (Figure 6.11). In addition, adding OGD SH-SY5Y cells demonstrated similar expression to the N9 only sample in both differentiated and undifferentiated cells (Figure 6.11). In addition, since equal numbers of SH-SY5Y cells were added to each well, this change is not due to reduced cell numbers following OGD. This suggests that the presence of neurons maintains low TREM-2 expression in microglia, that TREM-2 is increased following ischaemic injury of neurons and that differentiated neuronal cells have more capability to reduce TREM-2 expression.

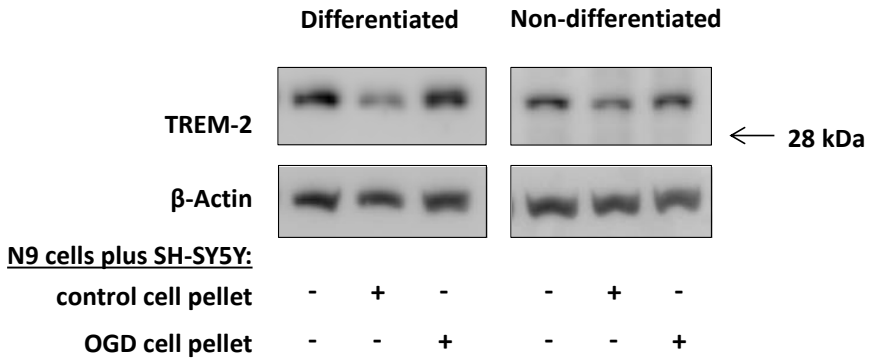

Figure 6.11: Addition of control but not OGD SH-SY5Y cells reduces TREM-2 expression in N9 cells. N9 cells were cultured in the presence or absence of control or OGD differentiated or undifferentiated SH-SY5Y cells. Cell pellets from 3 million SH-SY5Y cells were co-incubated with N9 cells for 4 h. Differentiated SH-SY5Y cells were differentiated with 10 µM all-trans retinoic acid for 9 days. TREM-2 protein expression was measured by western blot analysis, β-actin was used as loading control (n = 2).

To confirm these changes in TREM-2 expression in microglial cells, differentiated control or OGD SH-SY5Y cells were left in co-culture with N9 cells for 4 h, and following SH-SY5Y removal, TREM-2 expression measured by immunofluorescence using CFSE stain to label neurons. The absence of CFSE-stained cells (green) confirms the ability of the wash steps to remove SH-SY5Y cells and therefore all changes in TREM-2 expression were specific to the N9 cells. Figure 6.12 demonstrates that the addition of healthy (control) SH-SY5Y cells results in a reduction in TREM-2 expression in N9 cells compared to N9 cells alone. However, differentiated OGD-treated neuronal cells did not reduce TREM-2 expression, if anything, these cells increased TREM-2 expression in N9 cells (Figure 6.12). These results confirm that OGD treatment of SH-SY5Y cells inhibits their ability to reduce microglial TREM-2 expression.

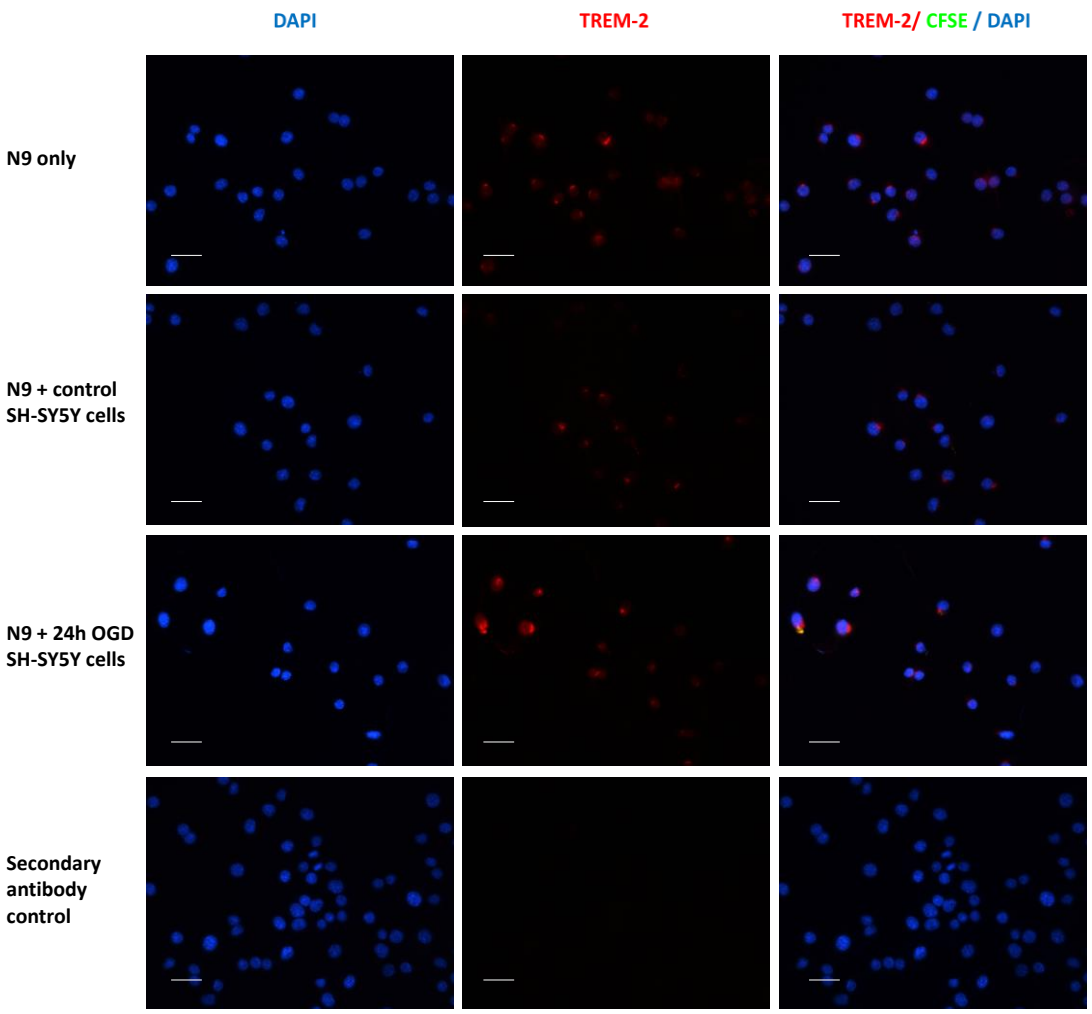

Figure 6.12: TREM-2 expression measured by immunofluorescence in N9 cells following incubation with OGD or control differentiated SH-SY5Y cells. Differentiated SH-SY5Y cells were incubated in control or OGD conditions and stained with CFSE (GREEN). N9 cells were cultured in the presence or absence of control or OGD differentiated SH-SY5Y cell pellets for 4 h. TREM-2 protein expression (RED) was analysed by immunofluorescence in permeabilized N9 cells. DAPI (BLUE) was used to stain the nucleus (n = 3).

6.5. Discussion

TREM-2 expression is increased in the brain following stroke and in other neurological conditions including neurodegeneration and MS [243, 352]. In the CNS, TREM-2 is mainly expressed in microglia and is involved in anti-inflammatory and phagocytic activities [81, 149, 240]. However, the mechanisms of TREM-2 upregulation in cerebral ischaemia are not known, therefore the aim of this study was to observe changes in TREM-2 expression in microglia following ischaemia by establishing an *in vitro* model of ischaemia. Interestingly, OGD had no direct effect on TREM-2 expression in N9 microglia in culture. However, using a co-culture system of neuronal and microglial cells, microglial TREM-2 expression was reduced by the addition of healthy control neurons but not OGD-treated neurons. No apoptosis occurred following OGD in the differentiated neuronal cells suggesting that this effect is not dependent on neuronal apoptosis. These results suggest that TREM-2 expression in microglia is generally low when surrounded by neurons but that damage or stress of neurons causes an increase in TREM-2 expression, likely to be due to the removal of an inhibitory signal that suppresses its expression in the presence of healthy neurons. This suggests that in situations such as stroke where neurons are subjected to ischemia, TREM-2 expression is increased.

Most studies demonstrate that TREM-2 is most highly expressed in microglia in the brain [81, 149], therefore these cells are likely to be the cells that express changes in TREM-2 expression following ischaemia. N9 microglia were exposed to OGD for 30 min or 2 h and then reperfused for up to 5 days, but OGD had no effect on TREM-2 expression in microglia at any time point. This suggests that regulation of TREM-2 in ischaemia requires other cells. This has been observed with other inflammatory mediators, for example the suppression of pro-inflammatory cytokine expression in microglia by apoptotic neurons [199]. In addition, TREM-2 expression in microglia has been previously shown to be altered by co-culture with mesenchymal stem cells through CX3CL1, further suggesting that TREM-2 expression in microglia is regulated by other cell types [375]. Neurons are the main cell type of the brain. The health of these cells is heavily monitored by microglia, and when neurons in the surrounding environment are damaged or stressed e.g. during ischaemia, microglia become activated [159]. In addition, the role of TREM-2 in the phagocytosis of apoptotic neurons led to the investigation of the effect of ischaemic neurons on TREM-2 expression in microglia [81, 530]. This study used the SH-SY5Y neuroblastoma cell line, a neuronal model that has been previously well characterised [527]. SH-SY5Y cells were left undifferentiated or differentiated with all-

trans retinoic acid to form a more neuronal cell type [524]. Both differentiated and undifferentiated SH-SY5Y cells have been tested in this study for their effects on TREM-2 expression in microglia. Undifferentiated SH-SY5Y cells are more stem-cell like and therefore may have different effects on TREM-2 regulation to differentiated SH-SY5Y cells [531, 532]. Stem cells have been previously shown to increase TREM-2 expression in microglia, and therefore the effect of these cells on TREM-2 expression was also of interest [375]. In addition, more stem cell-like neuronal cells are present in the repair phase following stroke when TREM-2 expression is also increased and therefore these cells may increase TREM-2 expression following stroke [375]. There was a significant reduction in cell viability, by MTS assay, following 24 h OGD in both differentiated and undifferentiated cells. On the other hand, the TUNEL assays showed that in undifferentiated cells, apoptotic cells were increased by 62.7%, but in differentiated SH-SY5Y cells, OGD had no significant effect. In addition, basal levels of TUNEL positive cells were higher in undifferentiated cells compared to differentiated SH-SY5Y cells, suggesting that undifferentiated cells in culture are more susceptible to cell death than differentiated cells. This suggests some discrepancy between the two assays. After further consideration, it is likely that the MTS assay showed a reduction in cell viability in both differentiated and undifferentiated cells because of the lack of oxygen and glucose in the media, as this will have slowed down the metabolism of the SH-SY5Y cells and so this assay was not used in future ischaemia experiments. On the other hand, the TUNEL assay binds to fragmented DNA produced in apoptosis, and therefore is the most reliable measure of cell death shown here. The TUNEL assay suggests that differentiated SH-SY5Y cells are resistant to OGD-induced apoptosis but undifferentiated cells are not. This finding confirms previous studies comparing differentiated and undifferentiated SH-SY5Y cells, which showed that differentiated cells are resistant to 1-methyl-4-phenylpyridinium and 6-hydroxydopamine-induced cell death but undifferentiated cells are not [524]. It is important to note that the TUNEL assay can also detect some necrotic cells, therefore future studies would aim to confirm the type of cell death that occurs here using other methods of apoptotic and necrotic cell detection including annexin V, active caspase 3 and propidium iodide [533].

After measuring the effects of OGD on SH-SY5Y cell death, these cells were co-cultured with N9 cells to observe their effects on TREM-2 expression. Adding the supernatants from either differentiated or undifferentiated SH-SY5Y cultures from control or OGD neurons had no effect on TREM-2 expression in N9 cells. However,

co-incubation of control SH-SY5Y cells only (without supernatant) with N9 cells showed a small reduction in TREM-2 expression in both differentiated and undifferentiated cells. Interestingly, adding OGD SH-SY5Y cells caused a small but consistent increase in TREM-2 expression compared to adding control SH-SY5Y cells. A moderate change in TREM-2 expression was seen when equal numbers (one million) of SH-SY5Y and N9 cells were incubated together for 4 h. However, many studies with microglia and neuronal co-cultures use neuron:microglial ratios between 2:1 and 5:1 [534, 535]. In addition, the ratio of microglia to neuronal cells throughout the brain is varied, and therefore this study analysed the effect of adding increasing numbers of undifferentiated SH-SY5Y cells on TREM-2 expression in N9 cells [536]. Increasing the number of neuronal cells enhanced the effect of these cells on the suppression of TREM-2 expression in microglial cells. This finding suggests that TREM-2 expression in microglia is partially regulated by the number of neurons present. This suggests that as neuronal cell numbers increase, the expression of TREM-2 in microglia in the region decreases. Forabosco and colleagues have shown that TREM-2 expression throughout the brain is varied, and that TREM-2 is highly expressed in particular brain regions including the hippocampus, medulla and substantia nigra [429]. In addition, expression of TREM-2 in the brain was mainly found in regions expressing low or no neuronal markers confirming observations seen here [429]. These initial studies suggest that TREM-2 expression is low in microglial cells in the presence of healthy neurons and that the absence of these cells, for example. N9 cell culture alone, allows a high expression of TREM-2 to be maintained that may not be observed in all areas of the CNS *in vivo*. This is an important point to consider when carrying out *in vitro* studies of TREM-2 expression in disease, particularly since individual microglial cell cultures have constitutively high levels of TREM-2 that would usually be suppressed in their natural environment. This may partially explain why TREM-2 expression is increased in many inflammatory diseases, yet so little is known about how this is regulated.

After optimising the ratio of neuronal:microglial cells required to observe an optimal change in TREM-2 expression (3:1), the effect of control and OGD neurons on microglial TREM-2 expression was studied by both western blot analysis and immunofluorescence. Both differentiated and undifferentiated control (healthy) SH-SY5Y cells reduced TREM-2 expression, and this effect was lost following OGD. The differentiated cells suppressed TREM-2 expression to a greater degree than undifferentiated cells. Although more experiments are required to validate these results, the difference observed after differentiation of SH-SY5Y cells may be

because expression of the molecules required for suppression of TREM-2 expression are increased on differentiated cells, including CD22 and CD47, which are cell surface antigens that suppress myeloid cell activation [144, 537]. Future studies will use immunofluorescence to view the changes in cell surface marker expression on SH-SY5Y cells following differentiation, focusing on molecules involved in suppression of microglial activation. This study suggests that regulation of TREM-2 expression in the animal model of stroke requires cell contacts between neurons and microglia to regulate TREM-2 expression. This hypothesis is confirmed by studies showing that healthy neurons express molecules on their cell surface including CD200 and CD47 that inform microglial cells that the neuron is healthy, activating signalling pathways in the microglial cell to inhibit microglial activation [142]. These signals are lost in damaged or stressed neurons, removing the suppression on microglial activation [142]. From this study, we hypothesise that TREM-2 expression in microglia is suppressed by the presence of healthy neurons and the removal of this suppressive signal following ischaemia causes an increase in TREM-2 expression. To confirm this hypothesis, future work will use cell culture well inserts to separate SH-SY5Y cells to allow them to be in culture together but not make cell contact.

The effects of differentiated and undifferentiated SH-SY5Y cells on TREM-2 regulation in microglia were compared. Differentiated SH-SY5Y cells had a greater ability to reduce TREM-2 expression in N9 cells than undifferentiated cells, which is most likely due to increased neuronal markers on the cell surface for regulation of microglial activity. However, when considering the mechanism of TREM-2 regulation in ischaemia, this study first hypothesised that the increase in TREM-2 expression was due to an increase in apoptotic cells due to the role of TREM-2 in phagocytosis. However, this study shows that this is not the case, as there was no significant increase in apoptotic differentiated SH-SY5Y cells after OGD. This suggests that in OGD, TREM-2 expression is increased by a change in markers on neuronal membranes triggered prior to apoptosis.

TREM-2 upregulation on day 7 in the MCAo mouse model of stroke and the anti-inflammatory and phagocytic functions of TREM-2 led us to hypothesise that TREM-2 was beneficial in stroke. Towards the end of this study, colleagues at the University of Jena confirmed that TREM-2 was upregulated 7 days following stroke in this model. However, using TREM-2 KO mice, they found that these mice had reduced inflammation in the brain measured by reduced microglial activation and IL-1α, IL-1β, TNF-α CX3CR1, CCL2 and CCL3 expression. These results suggest that

TREM-2 is pro-inflammatory in stroke [360]. TREM-2 deletion had no effect on infarct volume at either 7 or 28 days after occlusion, suggesting that the increase in inflammation did not affect stroke outcome [360]. This suggests that TREM-2 is increased in stroke which matches the observations from the *in vitro* model of ischaemia established in this study.

One key limitation of this study is that the work is performed in cell lines. To address this limitation, future work aims to isolate primary mouse neuronal and microglial cells to repeat key experiments. In addition, future studies will use mouse brain slices in culture to view the effect of ischaemia on TREM-2 expression in the brain slices, measured by immunofluorescence. The main limitation with slice culture is the duration of the culture required. *In vivo,* TREM-2 was increased 7 days after ischaemia, and if similar time points are expected *ex vivo*, the slices are unlikely to be healthy in culture for that length of time after dissection. Another limitation is the use of mouse:human co-cultures, although these co-cultures have been previously used [538-541], there may be some species differences, therefore future studies should use human or mouse only co-cultures to confirm these changes.

6.6. Summary

In summary, this study has established an *in vitro* model of ischaemia to study the role of microglia and neurons in the regulation of TREM-2 expression. This model shows that ischaemia simulation does not alter TREM-2 expression in microglial cultures alone, but that the increase in TREM-2 expression in ischaemia is likely to depend on neurons. The addition of healthy neurons reduced basal TREM-2 expression in microglial cells, which is confirmed by another report showing low TREM-2 expression in microglia in areas of the brain where high levels of neuronal markers are found [429]. In addition, this study suggests that TREM-2 expression is increased in the presence of ischemic neurons and therefore this may explain how TREM-2 is increased in stroke.

7. General Discussion and Future Directions

TREM-2 has been identified as an important mediator in the resolution of inflammation though its anti-inflammatory and pro-phagocytic functions [81, 240]. TREM-2 is also known to be increased in inflammatory diseases including COPD, rheumatoid arthritis and MS [242-244]. The expression of TREM-2 in myeloid cells is widely acknowledged, however, information regarding its expression in non-myeloid cells was limited. [81, 240, 262]. This study showed that TREM-2 expression is not restricted to myeloid cells types and may be expressed in some melanoma and epithelial cell lines and in primary cells of the lung. TREM-2 was expressed on ciliated bronchial epithelial cells of respiratory bronchioles in healthy and asthmatic lungs. TREM-2 expression was highly expressed on the cilia of these cells suggesting that TREM-2 may be involved in cilia function. Cilia are important for the clearance of debris and mucus from the lungs, and TREM-2 is also important for clearance of pathogens, therefore enhancing cilia function may be another way TREM-2 increases pathogen clearance [426]. The function of TREM-2 on these ciliated epithelial cells in the lung requires further research and may uncover new functions for TREM-2. To test this, an *in vitro* model of cilia movement will be developed as described by Milara and colleagues, where an air-liquid cell culture model is established using bronchial epithelial cells from human lung biopsies [542].

In myeloid cells, TREM-2 is downregulated by inflammatory mediators including IL-1β, TNF-α and LPS [241]. However, few inflammatory mediators have been shown to increase TREM-2 expression and there are no known signalling molecules shown to regulate induced expression of TREM-2. This study found that IL-4 but not IL-13 increases TREM-2 expression in monocytes and identified a novel mechanism of TREM-2 upregulation which required PI3K. In addition, the immunomodulatory cytokine TGF-β1 is identified as a novel inducer of TREM-2 expression in monocytes. Furthermore, TGF-β1-induced TREM-2 protein expression required PI3K, p38 MAP kinase and MEK1/ERK1/2 signalling pathways. This novel mechanism of TREM-2 regulation required PI3K and p38, but not ERK1/2 activity for TREM-2 gene expression, suggesting that ERK1/2 is required post-translationally for this increase in TREM-2 expression. This study has shown for the first time that PI3K, p38 and the ERK1/2 signalling pathways regulate induced expression of TREM-2 (Figure 7.1). Future studies will focus on the transcription factors required for TGF-β1-induced TREM-2 expression and study the post-translational mechanism by which the ERK1/2 signalling pathway mediates its effects on TREM-2 protein expression. In addition, the identification of IL-4 and TGF-β1 as mediators of TREM-2 expression, suggests that these mediators may be

required for the upregulation of TREM-2 expression observed in inflammatory conditions including allergic rhinitis, rheumatoid arthritis and MS where these mediators are upregulated [448, 543-546]. To test this hypothesis, future studies will investigate the effect of knocking out TGF-β1 and IL-4 on TREM-2 expression in animal models of inflammatory diseases. In addition, future studies will also investigate the effect of other inflammatory mediators on TREM-2 expression including a variety of chemokines and cytokines that were not tested in this study. These studies will also be carried out in macrophages and/or microglial cells, as these cells are the most likely to be present and have functional effects in inflamed tissues in inflammatory diseases.

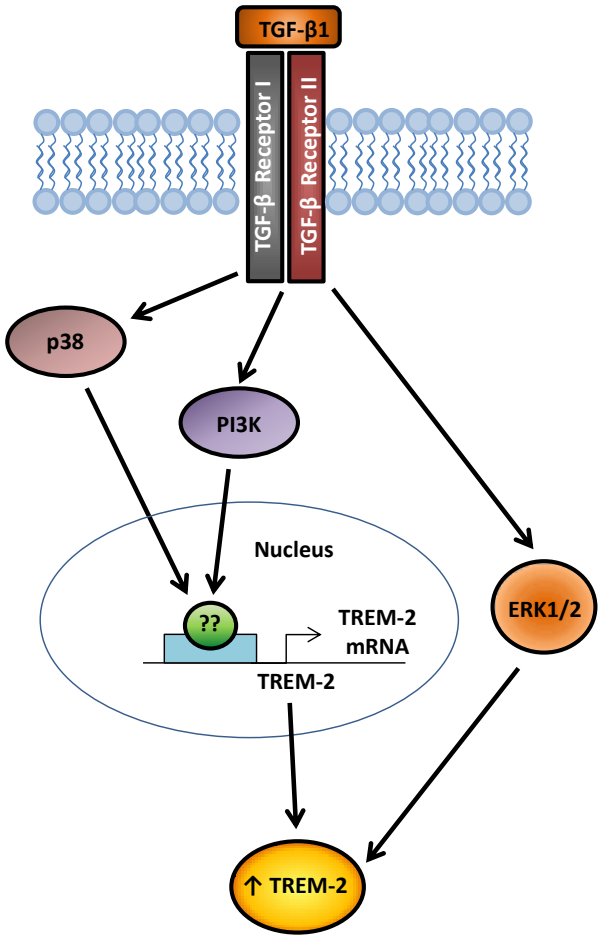

Figure 7.1: Mechanism of TGF-β1-induced TREM-2 expression.

This study also demonstrated a functional effect for TREM-2 in resolution of inflammation, as it was required for TGF-β1-induced MMP-1 expression in

monocytes. Interestingly, MMP-1, TGF-β1 and TREM-2 have all been shown to be important for efficient wound healing [33, 119, 334, 491]. MMP-1 and TGF-β1 increase angiogenesis and epithelial migration, two activities vital for wound healing [34, 466, 470, 513], and therefore, since TGF-β1-induced MMP-1 requires TREM-2, this may partially explain how these signalling molecules mediate their positive effects on wound healing. To test this hypothesis, future studies aim to investigate the effects of TREM-2 on epithelial migration and angiogenesis. Epithelial migration and angiogenesis will be measured using endothelial and epithelial scratch migration assays, measuring angiogenic factors including VEGF and FGF and using a 3D Matrigel model to analyse tube formation. In addition, this is the first time that TREM-2 has been linked to MMP-1 regulation and therefore this area requires further investigation. Future studies will investigate the effect of TREM-2 KD, over-expression and activation, on the expression and activity of a wide range of MMPs and their inhibitors (e.g. TIMPS) which may reveal new functions for TREM-2. These studies will be carried out in macrophages, as MMPs have a variety of functions in these cells which have been widely published and therefore can more easily be investigated [547-549].

Most studies suggest that TREM-2 has anti-inflammatory effects due to its ability to reduce pro-inflammatory cytokine secretion and increase anti-inflammatory cytokine secretion in a variety of models [81, 240]. However, a few studies have shown that TREM-2 may not be anti-inflammatory in all circumstances. For instance Correale and colleagues showed that KO of TREM-2 in an animal model of colitis reduced disease severity, pro-inflammatory cytokine secretion and bacterial killing suggesting that in this case, TREM-2 is pro-inflammatory [337]. Although this study has focused on the regulation of TREM-2 expression, the identification of IL-4 and TGF-β1 as inducers of TREM-2 expression provides us with more insight into the functions of TREM-2. IL-4 and TGF-β1 have both pro- and anti-inflammatory functions. IL-4 is increased in asthma and allergy and in these circumstances, increases inflammation [550]. However during rheumatoid arthritis and wound healing IL-4 is beneficial in promoting the resolution of inflammation [97, 551]. Similarly, TGF-β1 has many anti-inflammatory functions reducing inflammation in models of MS, rheumatoid arthritis and others [469, 476, 552]. However, in other conditions including pulmonary fibrosis, TGF-β1 can increase inflammation due to pro-fibrotic functions [553, 554], and since these diverse cytokines increase TREM-2 expression, it is likely that similarly to its inducers, TREM-2 is also anti-inflammatory in some environments and pro-inflammatory in others.

The anti-inflammatory mechanisms of TREM-2 have recently been studied in BMDMs showing that TREM-2 inhibits TLR induced ERK activation to supress inflammatory cytokine secretion (Figure 7.2) [297]. This finding was unexpected since TREM-2 activation has been previously shown to active ERK1/2 in myeloid cells [291]. This suggests that different mechanisms of TREM-2 activation result in different signalling pathways and functions. It may also be that activation of TREM-2 intracellularly results in activation of a different signalling cascade compared to if TREM-2 is activated by its ligand extracellularly.

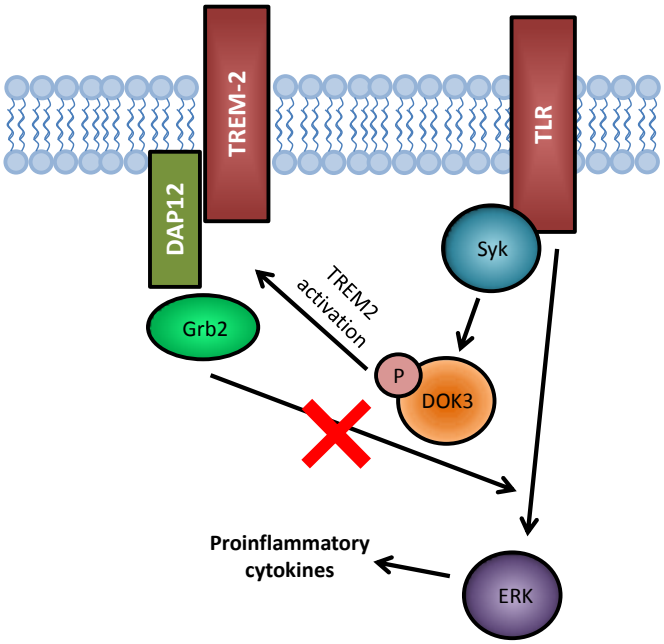

Figure 7.2: Proposed mechanism for TREM-2 suppression of TLR induced inflammatory cytokine secretion.

This pro- and anti- inflammatory effect is also demonstrated in the CNS. In MS, TREM-2 is anti-inflammatory [243]. However, in stroke, evidence from KO mice suggests that it is pro-inflammatory, therefore further information regarding the functions of TREM-2 in different conditions is required [360]. It is likely that TREM-2 function is regulated by mediators in the surrounding environment that differ between diseases, resulting in either pro- or anti- inflammatory effects depending on the condition. However, despite the contrasting roles of TREM-2 in inflammatory conditions, TREM-2 is consistently increased, for example in MS and stroke [278, 360]. However, the mechanisms of TREM-2 regulation in stroke are unknown. This study characterised an *in vitro* model of stroke using OGD to discover the mechanisms of TREM-2 upregulation in stroke. OGD alone was not sufficient to induce TREM-2

expression in N9 murine microglia, but the regulation of TREM-2 required the presence of neurons. Basal expression of TREM-2 in the microglial cell line was reduced in co-culture with the neuronal SH-SY5Y cell line, suggesting the presence of neurons supresses TREM-2 expression. This finding is confirmed by a recent study by Forabosco and colleagues showing high expression of TREM-2 in areas without neurons [429]. Interestingly, after OGD treatment of the neuronal cell line, co-culture with N9 microglial cells prevented the suppression of microglial TREM-2 by the neurons. This suggests that when neurons of the brain become ischaemic e.g. in stroke, the microglia in a neuron-rich environment express higher levels of TREM-2. High TREM-2 expression in these microglia may be important for priming of the cell for efficient phagocytosis of apoptotic cells and cell debris. The ability of healthy but not ischaemic neurons to reduce TREM-2 expression and the requirement of co-culture suggests that this event is mediated by changes in cell surface protein expression in neurons following ischaemia. Healthy neurons express signalling molecules on their surface including CD200 and CD47 that inform the surrounding microglia that the neuron is healthy [142, 519, 520]. However, when the neuron becomes stressed or damaged, these signals are lost and they also express other signals including ATP and together, this informs the microglia of the damaging environment, initiating microglial activation [523]. From this study we hypothesise that TREM-2 is reduced by a molecule on the surface of neurons including CD47 or CD200 and that the loss of this molecule or the gain of other molecules on the surface of the cell during ischaemia causes the upregulation of TREM-2 expression. To test this hypothesis, future studies will investigate changes in surface expression on neurons with ischaemia to understand the molecules required for TREM-2 regulation.

In conclusion, prior to this study there was little known about the expression of TREM-2 in non-myeloid cells. This pilot study suggests that TREM-2 may also be expressed in melanoma cells and on the cilia of airway epithelial cells, revealing potential new functions for TREM-2 in cancer and cilia function. TREM-2 expression was also confirmed in myeloid cells where it was located in the Golgi apparatus. In addition, few mediators were known to increase TREM-2 expression, and this study has revealed both IL-4 and TGF-β1 as key inducers of TREM-2 expression in monocytes, suggesting that these cytokines may contribute to increased TREM-2 expression in inflammatory diseases. The intracellular signalling pathways required for induced expression of TREM-2 were unclear, and therefore this study also demonstrated the involvement of PI3K, p38 MAP kinase and MEK1/ERK1/2 MAP kinase signalling pathways in TREM-2 regulation by TGF-β1 and IL-4. Furthermore,

TREM-2 was linked to the regulation of MMPs for the first time. Finally, the mechanisms of TREM-2 regulation in stroke were unknown and this study suggests that ischaemia-induced changes on neurons are required for the increase in TREM-2 expression on microglia following stroke. Together, this research has advanced the field of TREM-2 by revealing new mechanisms of regulation and expression which indicate new functions for TREM-2 that are yet to be discovered. This research further supports the role of TREM-2 in the resolution of inflammation but also indicates a role for TREM-2 in tissue repair. These results suggest that TREM-2 may have potential as a therapeutic target in inflammatory diseases.

8. Appendix

8.1. Buffers and Solutions

8.1.1. Fibronectin Coating:

Flasks were pre-coated with 2 mL coating media and stored at RT until use.

Coating media: 88 mL LHC basal medium, 10 mL 0.1% BSA, 1 mL Vitrogen and 1 mL human fibronectin.

8.1.2. qRT-PCR:

RT Master Mix: 2.5 µL 25 µM $MgCl_2$, 1 µL 10 x RT Buffer, 1 µL 2.5 µM dNTPs, 1µL 50 µM Random Hexomers (diluted 1/4 in analytical reagent grade water (Fisher Scientific)), 4 µL Reverse Transcriptase and 4 µL RNase inhibitor; per sample, all reagents were obtained from Applied BioSystems.

PCR Mix: 10 µL SYBR Green JumpStart Taq ReadyMix (20 mM Tris-HCl, pH 8.3, 100 nM KCl, 7 nM $MgCl_2$, 0.4 mM of each dNTP (dATP, dGTP, dCTP and dTTP), stabilisers, Taq DNA Polymerase, Jump Start Taq antibody and SYBR Green I), 4 µL RNase DNase free dH_2O, 1 µL F/R primer mix (5% Forward Primer (5 µM) 5% Reverse Primer (5 µM) in RNase DNase free dH_2O) per sample.

8.1.3. Western Blotting

General Western Blot Protocol Recipes:

Nuclear Cytosolic Fractionation Buffer A: 100 µL 1 M HEPES (pH 7.9) (Fisher), 100 µL 1M KCl (Fisher), 3 mL sucrose (Fisher), 15 µL 1M $MgCl_2$ (Fisher), 5 µL 1M DTT (Sigma), 10 µL NP-40 and 6.77 mL distilled water.

Nuclear Cytosolic Fractionation Buffer B: 200 µL 1 M HEPES (pH 7.9), 1 mL 1M KCl, 200 µL 5M NaCl, 5 µL, 1 M DTT, 2 mL glycerol (Fisher), 6.6 mL distilled water. Both buffers were stored at -20°C for future use.

4X LDS sample buffer (Life Technologies): diluted to 1X with PBS.

2X Novex Tris-Glycine SDS: Diluted to 1X using PBS.

20X TBST: 20 mM Tris base, 137 mM NaCl (Fisher), 1 M HCl (Fisher) and 0.1% Tween 20 (Fisher) in 1 L dH_2O to pH 7.6.

1X TBST: diluted in dH_2O from 20X TBST

Blocking solutions: 20 g Marvel milk powder plus 400 mL 1X TBST. Alternatively 1% BSA in 1X TBST was filtered and used for the MMP-1 antibody staining for all wash steps and primary and secondary antibody incubations.

1X Reblot Plus Strong Solution (Millipore Merck): Diluted 10X Reblot Plus Strong Solution one in ten in dH_2O.

Stripping solution ('in house' recipe): 15 g glycine, 1 g SDS, 10 mL Tween 20, top up to 1 L with dH_2O and make to pH 2.2.

Novex System:

10X Transfer buffer: 24.26 g tris base (Formedium, Norfolk, UK), 112.6 g glycine (Fisher) and 1 g SDS (Fisher) in 1 L dH_2O.

1X Transfer buffer: 100 mL 10X transfer buffer, 200 mL methanol (Fisher) and 700 mL dH_2O.

1X MOPS buffer: made from 20X by diluting in dH_2O (Life Technologies).

1X MES buffer: made from 20X by diluting in dH_2O (Life Technologies).

BIO-RAD System:

Resolving gel stock: 90.75 g tris base, top up to 500 mL with dH_2O and pH to pH 8.8 then add 2 g SDS.

Stacking gel stock: 12.11 g tris base, top up to 200 mL dH_2O and pH to pH 6.8 then add 0.8 g SDS.

10% SDS-PAGE Acrylamide Resolving Gel: 3.7 mL resolving gel stock, 5 mL 30% acrylamide (diluted from 40% in dH_2O, from Fisher), 6 mL dH_2O, 130 µL 10% APS (Fisher) (in dH_2O) and 13 µL TEMED (Fisher).

Stacking Gel: 600 µL of stacking gel stock, 1 mL 30% acrylamide, 4.25 mL dH_2O, 75 µL 10% APS and 7.5 µL TEMED

5X Running buffer: 30 g tris base, 140 g glycine, 5 g SDS (Sodium dodecyl sulphate) top up to 1 L with dH_2O.

1X Running buffer: 200 mL 5X running buffer, 800 mL H_2O.

Coomassie Blue stain: 0.25% Coomassie brilliant blue R (Sigma), 40% methanol, 7% acetic acid (Fisher) in ddH_2O.

Destain: 10% acetic acid, 40% methanol in dH_2O.

1X Transfer buffer: 3.03 g tris base, 14.41 g glycine, top up to 900 mL with dH_2O then add 100 mL methanol.

8.1.4. Immunohistochemistry Recipes:

4% Paraformaldehyde: 4 g PFA (Fisher) was dissolved in 100 mL dH_2O. The solution was warmed until the PFA dissolved.

10X PBS: 80 g NaCl, 2 g KCl, 14.4 g $Na_2HPO_4 \cdot H_2O$, 2.4 g $KH_2PO_4 \cdot H_2O$ (all from Fisher) at pH 7.4 and fill up to 1 L in Millipore H_2O.

1X PBS: diluted in dH_2O from 10X stock.

10X TBS: 90 g NaCl and 61 g Tris Base in 1 L dH_2O. Adjust pH to 7.6.

1X TBST: Dilute 10X TBS 1:10 with dH_2O and add 1 mL Tween 20 and mix well.

Citrate Buffer: 1.92 g Citric acid (anhydrous) in 1 L dH_2O, adjust to pH 6.0 then 0.5 mL of Tween 20 and mix well.

ImmPACT NovaRED Peroxidase Substrate (Vector Laboratories): To 5 mL of dH_2O, add three drops of Reagent one and mix well, add two drops of Reagent two and mix well, add two drops of Reagent three and mix well add two drops of the Hydrogen Peroxide Solution (reagent three) and mix well.

Blocking solution (to make 100 mL): add 5 mL of the serum (of the secondary antibody host species) to 95 mL PBS containing 0.2% Triton X.

8.2. Results

8.2.1. Cell Viability Assays

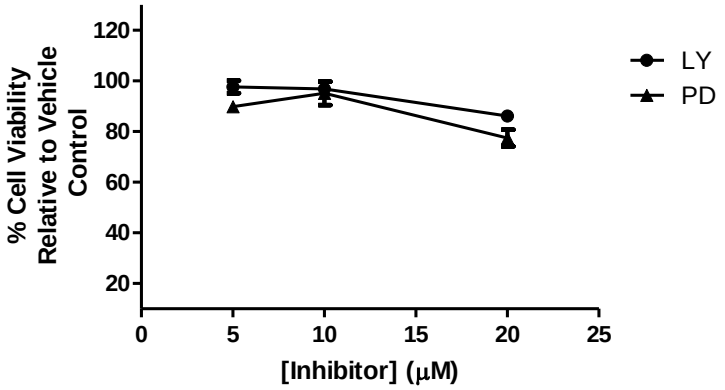

Figure 8.1: Effect of ERK1/2 and PI3K inhibitors on cell viability over 16 h in THP-1 cells. THP-1 monocytes were incubated with various concentrations of PI3K inhibitor LY294002 (LY), and ERK1/2 pathway inhibitor PD98059 (PD) for 16 h. Cell viability was measured by MTS assay, values show absorbance at 490 nm expressed as a percentage of the DMSO control. All inhibitors were dissolved in DMSO (n = 3).

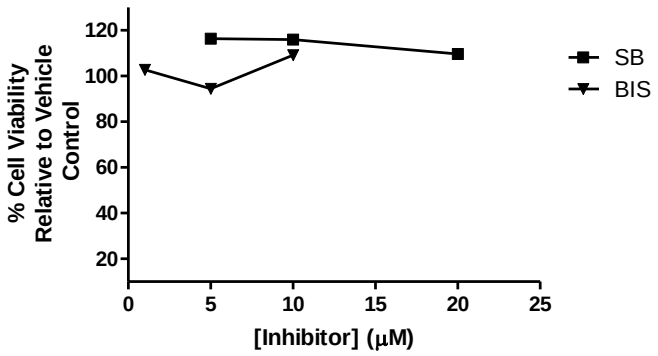

Figure 8.2: Effect of p38 and PKC inhibitors on cell viability over 16 h in THP-1 cells. THP-1 monocytes were incubated with various concentrations of the p38 MAP kinase inhibitor SB203580 (SB) and the pan-PKC inhibitor bisindolylmaleimide I (BIS) for 16 h. Cell viability was measured by MTS assay. Values show absorbance at 490 nm expressed as a percentage of the DMSO control. All inhibitors were made up in DMSO (n = 3).

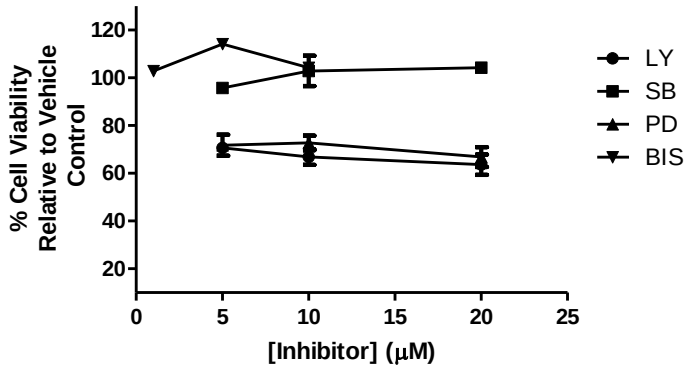

Figure 8.3: Effect of cell signalling inhibitors on cell viability over 48 h in THP-1 cells. THP-1 monocytes were incubated with various concentrations of the PI3K inhibitor LY294002 (LY), ERK1/2 pathway inhibitor PD98059 (PD), MAPK/p38 pathway inhibitor SB203580 (SB) and the PKC inhibitor bisindolylmaleimide I (BIS) for 48 h. Cell viability was measured by MTS assay, values show absorbance at 490 nm expressed as a percentage of the DMSO control. All inhibitors were made up in DMSO. MTS samples run in triplicate (n=1).

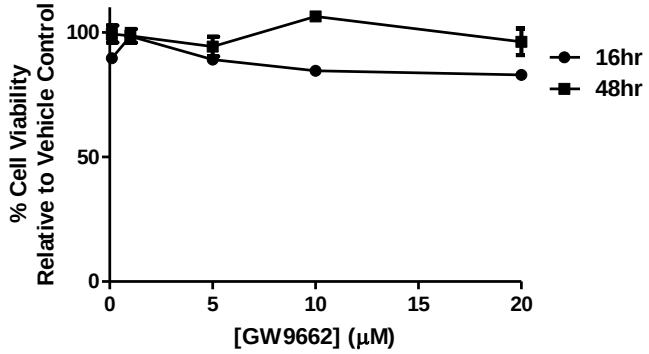

Figure 8.4: Effect of GW9662 on cell viability in THP-1 cells. THP-1 cells were incubated with the PPARγ inhibitor GW9662 (GW) (A) at 0.1, 1, 5, 10 or 20 μM for 16 or 48 h and cell viability measured by MTS assay. Cell viability values are absorbance at 490 nm and expressed as a percentage of the DMSO control. GW9662 was made up in DMSO, samples in triplicate (n = 1).

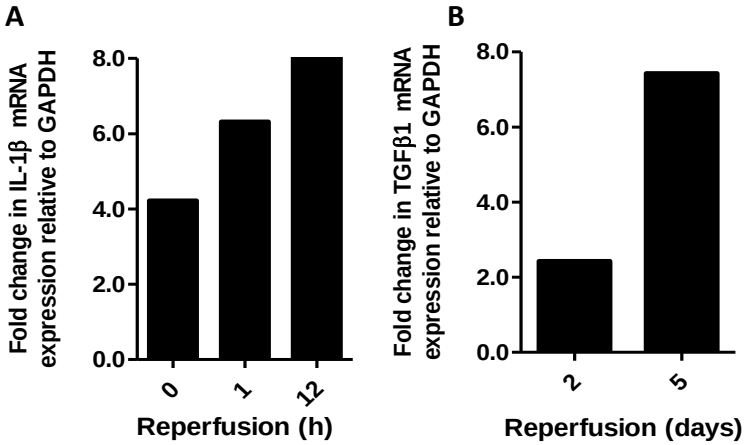

Figure 8.5: Hypoxia increases IL-1β and TGF-β1 in N9 murine microglial cells. N9 microglial cells were subjected to 6 h hypoxia or control conditions and reperfused for (1 h, 12 h, 2 days or 5 days) or not-reperfused (0 h) and samples taken for qRT-PCR analysis. IL-1β and TGF-β mRNA expression was measured by qRT-PCR. mRNA values are expressed as fold change relative to the non-hypoxic control and normalised to GAPDH (n = 1).

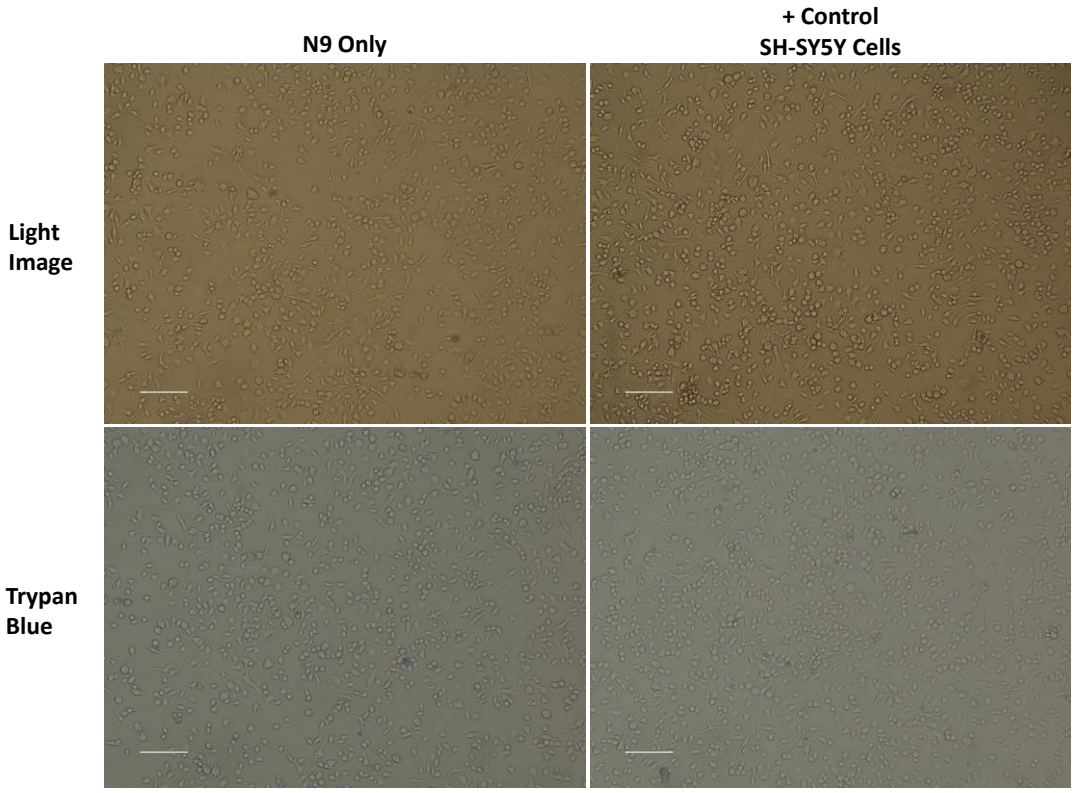

Figure 8.6: Effect of SH-SY5Y cells on N9 cell viability. 3 x 10^6 undifferentiated SH-SY5Y cells were added a 0.4 µm culture insert into a transwell containing 1 x 10^6 N9 cells and incubated for 4 h. After incubation the N9 cells were viewed under the microscope and stained with Trypan Blue to measure dead cells. Scale bar = 200 µm.

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