UNIVERSITY OF BIRMINGHAM

The role of amphoterin-induced gene and open reading frame (AMIGO) family in myelination and neurodegeneration

and

Investigating the roles of mesenchymal and hematopoietic stem cell conditioned media on neutrophil recruitment in a model of inflammation

by

Joseph Tickle

A thesis submitted to the University of Birmingham for the degree of

Masters of research (MRes)

School of Clinical and Experimental Medicine
College of Medical and Dental Sciences
The University of Birmingham
May 2013

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This project is submitted in partial fulfilment of the requirements for the award of the MRes

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Abstract

Myelination plays a key role in both the developing CNS and repair from neurodegenerative diseases such as multiple sclerosis (MS). Previous work has suggested a role for the amphoterin-induced gene and open reading frame (AMIGO) family of proteins, like other members of the leucine-rich repeat immunoglobulin domain (LRRIG) superfamily, in repair within the CNS. This thesis proposes a novel role for the AMIGOs in both the developmental and repair processes of myelination. The immunofluorescent staining results presented here show that the AMIGOs are expressed in the developing mouse brain in a similar, progressive pattern to that of myelination up to at least day 28. These results also show that the AMIGO proteins are expressed by oligodendrocyte precursor cells (OPCs), mature oligodendrocytes and astrocytes in the spinal cord during experimental autoimmune encephalomyelitis (EAE), an animal model of MS. These cells play a pivotal role in myelination, as either myelinating cells or regulators. The knockdown of AMIGO3, however, was shown not to affect OPC differentiation. These combined results suggest a role of this family of proteins as potential positive regulators of both myelination during development and remyelination post-disease.

Acknowledgements

I would like to thank Dr Zubair Ahmed for providing me with the opportunity to work in his lab and for his invaluable advice and guidance throughout. I'd also like to thank the other members of the Neurotrauma and Neurodegeneration group for their technical assistance. My thanks also go to the Medical Research Council for providing funding for this work.

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1. Introduction

1.1 Myelination and oligodendrocytes

Myelin is an electrically insulating material that coats the axon of neurons to form the myelin sheath. It is synthesised by mature oligodendrocytes, a type of glial cell, shortly after birth in mammals. Oligodendrocytes are formed in the neuroepithelium of the spinal cord and subsequently migrate within the CNS. Oligodendrocytes differentiate from oligodendrocyte progenitor cells (OPCs) within CNS tissues. OPCs constitute 8-9% of white matter of rat brain (Dawson et al., 2003). Each oligodendrocyte extends processes, which make contacts with 20-40 axonal segments via surface receptors. Signals from these receptors trigger myelin to form as an extension of the oligodendrocyte cell membrane, which wraps repeatedly around axons to form the sheath as seen in figure 1 (Hildebrand et al., 1993; Umemori et al., 1999). The gaps between these structures form the nodes of Ranvier. The myelin sheath gives axons a white appearance, hence “white matter”, and comprises the most abundant membrane structure of the vertebrate nervous system.

The primary function of myelin is to allow high-speed nerve propagation with a minimum amount of energy expenditure. This is achieved by its dielectric properties primarily due to its high protein content (around 80% dry weight). Around 30% of this protein is myelin basic protein (MBP), a functionally important structural protein (Umemori et al., 1999), while other proteins include the myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP).

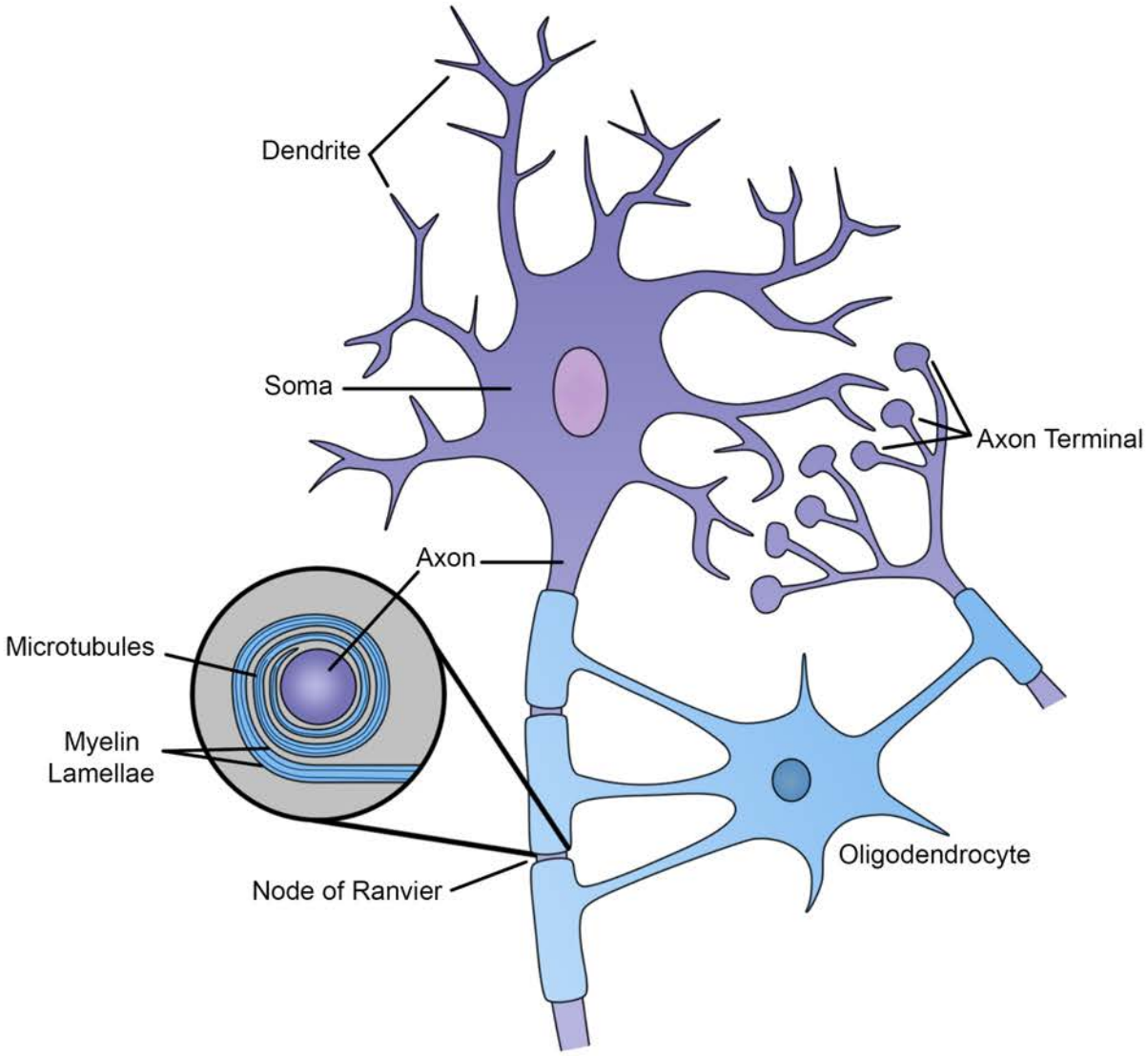

Figure 1 Ensheathment of an axon by oligodendrocytes The cell membrane of an oligodendrocyte extends and enfolds the axon with a distinct protein and lipid composition to form the myelin sheath. Microtubules, stabilised by MBP, give structure and allow transport of RNA, mRNA and ribosomes transport to distal sites of protein synthesis.

The process of myelination starts within the embryo and relatively late on in the development of the CNS, but can continue for a much longer time postnatally depending on the animal. Developmental myelination occurs in humans up until adolescence or beyond, particularly in regions such as the corpus callosum and corticospinal tract (Paus et al., 1999). Infant brain myelination is seen to occur asymmetrically; with myelination of the left-brain occurring more rapidly than that of the right, although the opposite is observed in the cerebellum (Deoni et al., 2011).

Without myelin, the conduction of the action potential can be severely impaired, resulting in paralysis and numbness such as that observed in diseases like multiple sclerosis (MS). The loss of the trophic support provided by the oligodendrocytes further predisposes axons to more severe injury (Trapp et al., 1998).

1.2 Demyelination and oligodendrocyte destruction in MS

MS is a sporadic, acquired, inflammatory disorder of the human brain and spinal cord. MS affects upwards of 2.5 million people worldwide, with the usual onset of symptoms in a person's late 20's to early 30's (Ransohoff, 2012). Disease progression is normally characterised by episodes of sporadic neurological deficits and periods of substantial remission; known as the relapsing-remitting stage, illustrated in figure 2. Between 8-20 years post onset, sufferers often enter the secondary progressive stage; characterised by severe, irreversible neurological disability (Trapp et al., 1999). These neurological deficits are heterogeneous and may involve any part of the central nervous system (CNS), however, half of all patients require aid while walking 15 years after the onset (Weinshenker et al., 1989). On average, life expectancy of MS sufferers is reduced by around ten years due to the chronic debilitating nature of the disease, defining MS as a leading cause of non-traumatic disability among young adults (Brønnum-Hansen, 2004; Noseworthy et al., 2000).

The primary cause of neurological deficits in MS sufferers is demyelination and destruction of oligodendrocytes, neurons and axons. This is caused by the invasion of the CNS by immune cells, which target the myelin sheath. MS has therefore been classically defined as an autoimmune disease, however the evidence for this is weak and circumstantial (Wootla et al., 2012). MS is now increasingly being recognised as a neurodegenerative disease triggered by an inflammatory attack of the CNS. There is no known definitive cause for MS; as such it is simply classified as a progressive, episodic, demyelinating disorder of the CNS.

Entry into the secondary progressive stage of MS is due to accumulation of axon degeneration. This may be myelin-dependent through altered ion channels, or myelin-

independent through perturbed mitochondrial function, reduced ATP/ADP balance and subsequent reduced axonal transport and axonal swellings (Nave, 2010). This irreversible degeneration correlates with permanent neurological disability.

Current MS treatments are largely immunosuppressive, with the use of drugs such as methylprednisolone and interferon alpha, with no oligodendrocyte-based treatment. These treatments passively treat oligodendrocytes and myelin destruction by modulating the inflammatory response. Although they appear to reduce the severity of symptoms and the frequency of relapse, they do not treat the pathological progression of the disease. The protection of oligodendrocytes and myelin or reversal of demyelination and axonal loss, and therefore progression from relapsing-remitting to secondary progressive MS, may therefore be of clinical significance.

An increase in the migration of a heterogeneous population of immune cells and chemicals across the blood-brain barrier is thought to be the primary trigger of MS. Regulatory defects within immune cells result in an immune response occurring within the CNS, primarily against myelin and oligodendrocytes. This inflammation is the first of four pathological features of the disease; followed by demyelination, axonal damage and reactive gliosis (Brück, 2005).

Once an immune response has been set up within the CNS, there are four main mechanisms, both biochemical and cellular, through which myelin and oligodendrocytes may be destroyed (Brück, 2005). B cell antibody-mediated autoimmune demyelination is the most frequently

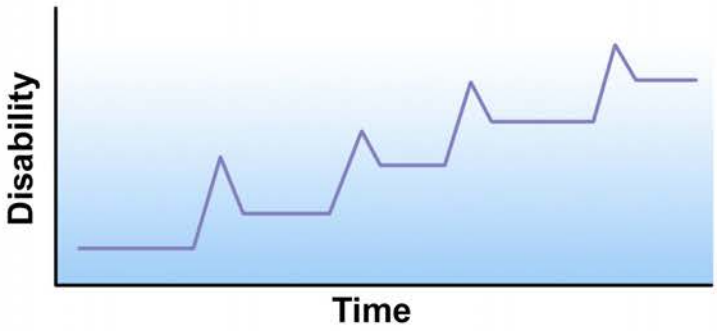

Figure 2 Relapse-remitting MS disease progression Unpredictable attacks of varying severity, which may or may not leave permanent deficits in ability followed by periods of remission.

observed pathology, occurring in over half of MS sufferers (Brück, 2005). The release of antibodies by B cells can result in the activation of complement and macrophages, leading to opsonization and phagocytosis. Another major pathology is that of T cell-mediated autoimmune demyelination; either the direct binding of T cells to epitopes on the myelin sheath, or the release of cytotoxic cytokines and soluble toxic mediators. Distal oligodendrogliopathy and initiation of the apoptotic cascade may also occur after non-lethal damage to oligodendrocytes as well as primary oligodendrocyte degeneration triggered by viruses and environmental toxins if the cells are weak after myelin damage.

The focal tissue damage forms multiple characteristic sclerotic plaques within the white matter of the CNS (Brück, 2005). These hallmarks represent the end stage of the degenerative process, from inflammation to neuronal and axonal degeneration.

1.3 Remyelination

The characteristics of relapsing-remitting MS are the degeneration of myelin, oligodendrocytes and axons and subsequent repair of this damage to varying degrees. During this process the inflammatory site changes from destructive to remyelinating despite the presence of immune cells (Ozawa et al., 1994). Although most active during the acute stages of the disease this process also occurs in the progressive phase, around 20% of lesions are fully repaired to form shadow plaques, defined as focal areas with reduced myelin density (Compston and Coles, 2008; Barkhof et al., 2003, Patrikios et al., 2006)

The recapitulating hypothesis suggests that remyelination occurs via the same mechanism as developmental myelination (Fancy et al., 2011). Importantly, it has also been suggested that the process of remyelination as a repair mechanism may differ from that of developmental myelination (Arnett et al., 2004). Whether the recapitulating hypothesis is correct or not, certain similarities are at least agreed on between the two processes. As with developmental myelination, oligodendrocytes play a fundamental role in the endogenous repair mechanism. Oligodendrocytes exhibiting an immature phenotype have been

documented in recently formed sclerotic plaques, suggesting that the oligodendrocytes involved in remyelination are newly differentiated (Wolswijk, 1998a). There is also a positive correlation between the number of oligodendrocytes in freshly formed lesions and the length of survival (Prineas et al., 1993). The presence of OPCs is therefore presumed to provide new oligodendrocytes that can remyelinate the naked axons within the lesion (Compston and Coles, 2008).

The extent to which plaques are remyelinated is highly heterogeneous, both between patients and between plaques found in the same patient (Patani et al., 2007). However, over time remyelination ceases to occur. The cessation is not due to a lack of OPCs as these cell populations have been found (Wolswijk et al., 1998b) suggesting impairment of the differentiation and proliferation processes of the OPCs. The reason for this effectively exhausted repair may be due to an absence of a growth factor, the presence of an inhibitory molecule or the build up of scar tissue (Woswijk, 1998b). If the cause can be elucidated, it may prove an important therapeutic target.

1.4 Experimental autoimmune encephalomyelitis (EAE)

EAE is an inflammatory demyelinating disease of the CNS (secondary to severe inflammation), used as an animal model of brain inflammation. The disease is induced by systemic injection of CNS-antigens and adjuvant, such as those to MBP, in order to induce an immune response specific to the CNS. It is the most widely used model to study MS as they share both clinical and pathological features (Lassmann, 1983). The form most similar to the relapsing-remitting stage of MS is seen in the chronic relapsing form (CREAE) exhibited by the Biozzi ABH mouse (Biozzi et al., 1972).

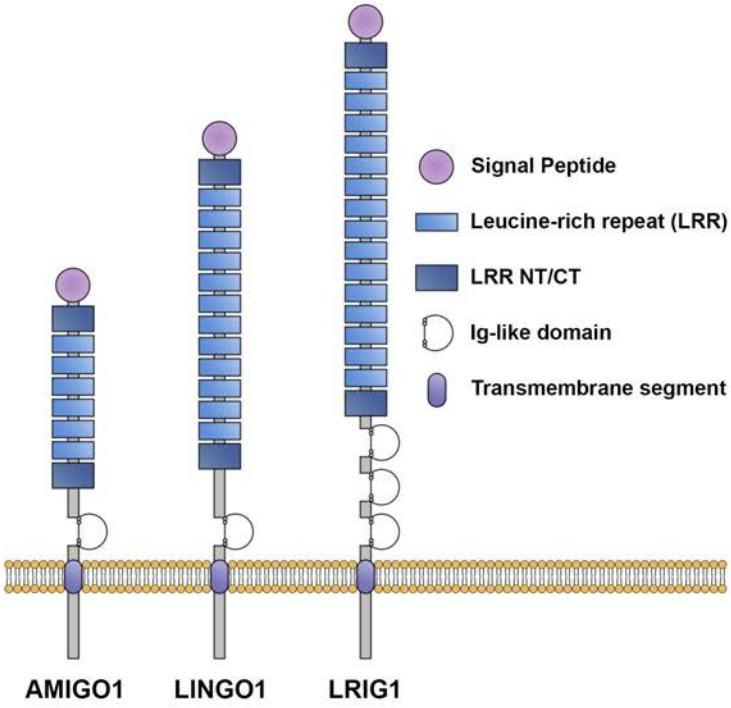

Figure 3 Domain organisation of LRRIG proteins A schematic representation of three LRRIG proteins: AMIGO1, LINGO1 and LRIG1. Each share very similar structures, consisting of a transmembrane segment, at least one Ig-like domain, varying numbers of LRRs and a signal peptide.

However, as with any animal model, there are limitations. For example it has been suggested that a different subtype of T-lymphocytes drive the disruption of the blood-brain barrier via secretion of a different array of interleukins than during MS (Compston and Coles, 2008). The use of potent adjuvant to initiate the disorder may bypass some fundamental stages in MS initiation and progression (Caprariello et al., 2011). However, it is still considered a highly similar and relevant model, particularly for the study of remyelination.

1.5 The LRRIG domain and LINGO1

The leucine-rich repeat (LRR) and immunoglobulin (Ig) domains are two discrete motifs, which differ in function and are found in 3% and 0.9% of all proteins in the human proteome, respectively (Pruess et al., 2003). The LRR domain was first found in the leucine-rich α2-glycoprotein and is involved in protein-protein recognition processes. It is a sequence of 11 conserved aliphatic amino acids, including leucine. The Ig domain consists of two anti-parallel β-sheets with a Greek key topology. Currently, there are 36 known proteins containing at least one copy of each of these motifs, known as LRRIG proteins, which can be further classified into 13 subgroups (Homma et al., 2009). Around two thirds of these LRRIG

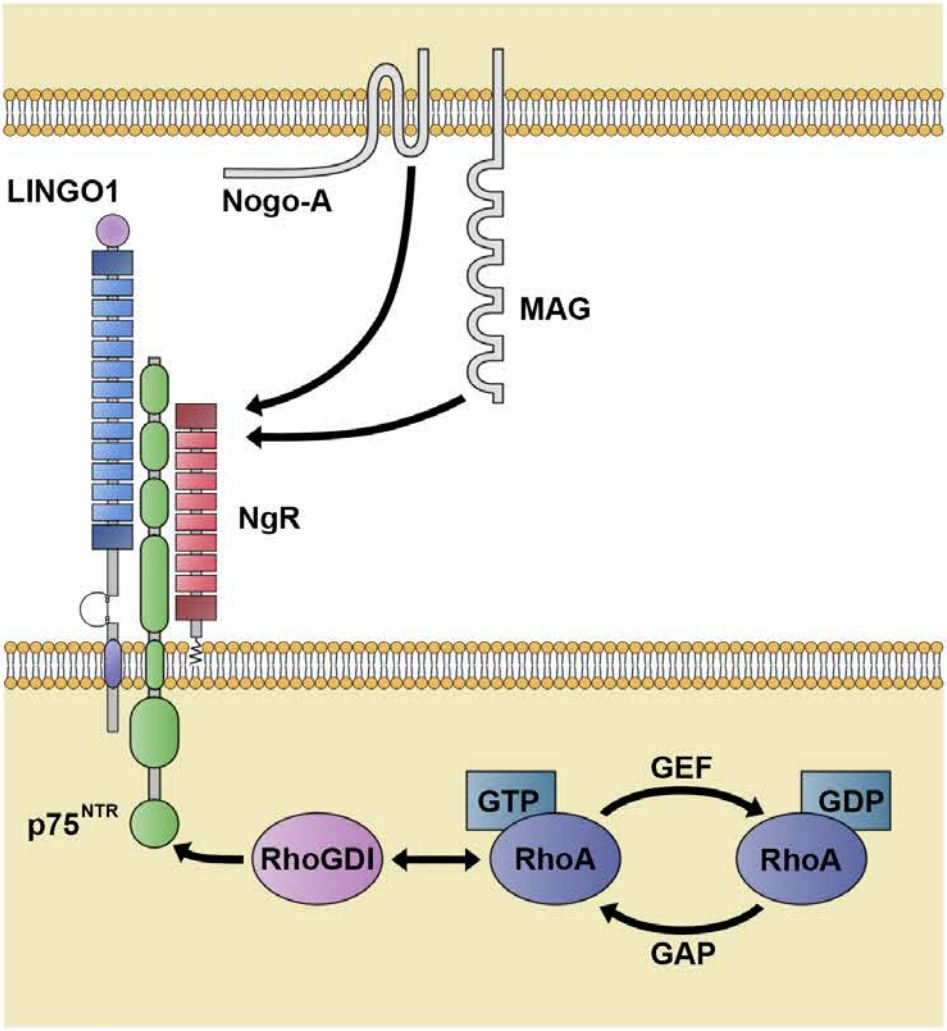

Figure 4 The LINGO1/NgR/p75 receptor complex The inhibitors Nogo-A and MAG intercellularly bind to the LINGO1/NgR/p75 neurotrophin signaling complex. On ligand binding, the RhoGDI-RhoA complex binds to p75, causing a strong inhibition of RhoGDI. This permits a guanine exchange factor (GEF) to perform GDP-GTP exchange leading to active stabilisation and subsequent inhibition of neurite outgrowth.

proteins are expressed within the central nervous system, including the LRR and Ig domain-containing, Nogo-receptor interacting protein 1 (LINGO1).

LINGO1, a member of the LINGO subgroup, is a highly conserved membrane-bound protein of 614 amino acids. As seen in figure 3, it consists of 12 LRR motifs, a single Ig-like domain, a transmembrane domain and a short cytoplasmic tail (Mi et al., 2004). LINGO1 is a co-receptor of the neuronal Nogo-66 receptor (NgR)/p75 neurotrophin signaling complex (figure 4). Components of myelin, such as MAG and Nogo, stimulate this receptor complex, in turn

activating RhoA and signaling the collapse of neural growth cones (Ahmed et al., 2006). LINGO1 expression in oligodendrocytes has also been shown to negatively regulate myelination. One such mechanism that has been suggested is also by activation of RhoA and the subsequent inhibition of OPC differentiation to mature myelinating oligodendrocytes (Mi et al., 2005). However, recent work suggests that LINGO1 has a dual function as a receptor and a ligand, a homophilic intercellular interaction between which mediates OPC differentiation and myelination (Jepson et al., 2012). LINGO1 has therefore been suggested as a potential target for controlling CNS myelination in disorders such as MS. EAE-induced LINGO1 knockout mice, as well as those treated with an anti-LINGO1 antibody, have shown marked stabilisation and reversal of disease progression as well as improving axonal health and remyelination (Mi et al., 2007).

1.6 The three AMIGOs

One family of three LRRIG proteins is the amphoterin-induced gene and open reading frame (AMIGO) subgroup, also known as the Alivin's, which exhibit similar structures and extensive sequence homology of around fifty percent (Kuja-Panula et al., 2003). All three are type I transmembrane proteins with similar structure to that of the LINGO1, seen in figure 3; a single Ig-like domain, an adjacent transmembrane domain and a short cytoplasmic tail, however the AMIGOs consist of only 6 extracellular LRR domains. Similarly to LINGO1, the family are hypothesised to function as cell adhesion molecules due to their homophilic and heterophilic binding capabilities (Chen et al., 2006). However, the AMIGOs are known to homodimerise via their LRR regions in order for proper cell surface expression (Kajander et al., 2011).

AMIGO1 is an N-linked glycosylated member of the family (Chen et al., 2012) that was originally identified by its upregulation in rat hippocampal neurons when exposed to the neurite outgrowth promoting protein amphoterin (HMGB1) (Kuja-Panula et al., 2003). It has been suggested that AMIGO1 is important in the survival/proliferation mechanism of HMGB1

in zebrafish, and is therefore important in forebrain development. Expression has been shown to increase throughout development to a maximum that is sustained into adulthood, suggested both a developmental and adult role of the protein (Chen et al., 2012). Unlike AMIGO2 and 3, AMIGO1 is expressed exclusively within the CNS within astroglia, oligodendroglia and neurons. Although predominantly dendritic, the removal of the LRR domains results in the localisation of AMIGO1 to the axon (Chen et al., 2012). Similarly to LINGO1 and other LRRIG proteins, substrate-bound AMIGO1 has been shown to be involved in neurite outgrowth in cultured neurons (Kuja-Panula et al., 2003), a depletion of AMIGO1 has also been shown to inhibit neurite outgrowth (Chen et al., 2012). The expression of AMIGO1 has also been shown to be neuroprotective *in vitro* (Chen et al., 2012).

AMIGO2 was first identified as Alivin-1 (after “alive” and “activity-dependent leucine-rich-repeat and Ig superfamily survival-related protein.”) for its involvement in depolarisation-dependent survival of cerebellar granule neurons (Ono et al., 2003). Although AMIGO2 is enriched within the hippocampus (particularly Cornu Ammonis 1 and 2, which are particularly resistant to neuronal injury and neurotoxicity) it is also enriched within the cerebellum, retina, liver and lung. It has been implicated in human gastric adenocarcinoma patients (Rabenau et al., 2004) as well as mental retardation (Gimelli et al., 2011).

The third member of the AMIGO family, AMIGO3 is particularly understudied, although it is ubiquitously expressed with no particular expression patterns (Kuja-Panula et al., 2003). Recently, it was shown that AMIGO3 can substitute for LINGO-1 and interact with NgR and p75 to signal growth cone collapse (Ahmed et al., 2013b). Knockdown of AMIGO3 in cell culture demonstrated enhanced dorsal root ganglion neuron neurite outgrowth suggesting a direct function in regulating CNS axon regeneration (Ahmed et al., 2013b). It’s involvement in MS has also been shown previously suggested within this lab (Ahmed, unpublished data).

1.7 Aims and hypotheses

The aim of this study was to investigate the involvement the AMIGO family of proteins in both developmental myelination and remyelination during disease. Specifically;

- To investigate AMIGO expression patterns compared to that of myelination during development.
- To examine AMIGO expression in OLs, OPCs and astrocytes during disease progression of EAE.
- To investigate the effects on differentiation of decreasing AMIGO3 expression in oligodendrocyte progenitor cells.

The major hypothesis of this thesis is that these AMIGO proteins, particularly AMIGO3, are expressed by oligodendrocytes and astrocytes and promote OPC differentiation and subsequent myelination of axons.

2. Materials and Methods

2.1 Animals

6-8 week-old female Biozzi ABH mice (Biozzi et al., 1972) were kindly provided by Professor David Baker (Queen Mary University of London). Four experimental groups were used each consisting of 6 mice, hence a total of 24 mice were used. The groups were defined as: 1), healthy controls without EAE; 2), mice at the acute stage of EAE; 3), mice at the first symptomatic relapse of EAE; 4), mice at the chronic stage of EAE.

2.2 EAE induction

All disease induction methods were licensed by the UK Home Office and approved by Queen Mary University of London Ethical Committee. Procedures were performed by Professor David Baker. EAE was induced as previously described by Baker et al., 1990 (Baker et al., 1990). Briefly, mice were injected subcutaneously into the hindflanks with mouse spinal cord homogenate dissolved in Freund's adjuvant, on day 0 and day 7. Animals were then weighed and monitored daily for their neurological signs. EAE was scored using a 5-point scale (Baker et al., 1990) where 1 = tail paresis, 2 = loss of gait, 3 = complete hindlimb paralysis, 4 = hindlimb/forelimb paralysis, 5 = death.

2.3 Tissue preparation

Animals were killed by CO_2 narcosis and perfused intracardially using 4% paraformaldehyde. Mice in the acute EAE group were sacrificed during the peak of initial disease (onset of complete hindlimb paralysis) at 17 days after disease induction, animals in the relapse group were sacrificed at the peak of relapse (day 37 when complete hindlimb paralysis), chronic EAE animals were killed at day 65, during which there was hind limb paralysis but no recovery.

2.4 Luxol fast blue (LFB) stain

Sections were washed in PBS and stained with LFB (Thermo Scientific, UK) solution (0.01% w/v in 95% ethanol with 10% acetic acid) at 55°C for 16-24h. The sections were then rinsed

in 70% ethanol, differentiated in 0.05% lithium carbonate solution for 4min followed by 70% ethanol and stopped with distilled water. Sections were dehydrated with ethanol (70%, 90%, 100%, 100%), cleared in Histoclear (National Diagnostics, Hull, UK) and mounted with Vectamount, all at RT (Vector Laboratories, Peterborough, UK).

2.5 Immunohistochemistry (IHC)

Monoclonal antibodies against AMIGO1-3 were from Santa Cruz Biotechnology, Santa Cruz, USA (SC-49879, SC-46760, SC-49881); antibody to GFAP was from Sigma, Poole, UK (G9269); antibodies to NG2, CaII and MBP were from Abcam, Cambridge, UK (ab101807, ab124687, ab40390). Alexa 488-conjugated secondary antibody was from Invitrogen, Paisley, UK (A11055) and Texas Red from Invitrogen (T6391).

Post-natal BALB/c mice (0, 7, 14, 21 and 28 day old) 15µm-thick brain tissue coronal sections and 15µm-thick spinal cord tissue sections were fully thawed, washed in PBS and permeabilised in PBS containing 0.1% Triton X-100 for 10 min at room temperature (RT). After further washes in PBS, sections were blocked in PBS containing 3% BSA and 0.05% Tween-20 for 30 min at RT in a humidified chamber. Sections were then incubated with the indicated primary antibodies (1mg/ml) overnight at 4°C (16-18 hr). After washes in PBS, sections were subsequently incubated with the appropriately labeled secondary antibody for 1h at room temp before mounting in VectaShield with DAPI (Vector Labs, CA, USA) and visualised using a Zeiss fluorescent microscope equipped with an Axiocam HRC, run through Axiovision Software (all from Zeiss, Hertfodshire, UK). RGC5 cultures were treated similarly but with primary antibody incubation adjusted to 1h. Negative controls were used in each run, which included omission of primary antibody and were used to set the background threshold level prior to image capture.

2.6 Cell culture

Retinal ganglion cell line (RGC5), initially thought to be RGC from rat retina but later shown by several investigators, including the MNG that the cells were oligodendrocyte precursor

cells, were grown in 75cm^2 culture flask in medium consisting of Dulbecco's Modified Eagle Medium (Invitrogen) containing 5mM Glucose, supplemented with foetal calf serum (10% $^{v}/_{v}$; Invitrogen), penicillin and streptomycin (10% $^{v}/_{v}$; Invitrogen) and 2mM L-glutamine (Invitrogen) in a humidified incubator at 37°C/5% CO_2.

2.7 siRNA-mediated knockdown

siRNA transfection was carried out using AMIGO3 sense siRNA (5'-TGGAGGAGCTGGAGAAGTT-3' (Dharmacon, CO, USA) with Lipofectamine 2000 (Invitrogen) according to the manufacturers protocol and routinely used in the MNG laboratory (Ahmed et al., 2011; Douglas et al., 2009; Ahmed et al., 2013). siRNA (20pmol) was introduced to each well of a 8-well chamber slide containing RGC5 cells at 5x10^3 cells/well in non-supplemented DMEM (Invitrogen) and incubated for 5h at 37°C/5% CO_2. Complete RGC5 medium was then added and cells incubated for 48h at 37°C/5% CO_2 before IHC staining was performed to validate immunohistochemical levels of knockdown.

3. Results

3.1 The brain is progressively myelinated during development

To determine myelination patterns during normal development, studies were conducted using primary mouse brain slices from birth to 28 days. Both a traditional staining approach, Luxol Fast Blue (LFB), and immunohistochemistry were performed to elucidate myelination patterns within the developing brain of postnatal mice; a well documented and recognised process.

The LFB staining revealed typical progressive myelination of the mediolateral corpus callosum of mouse coronal sections from p0 to p28 (figure 5). Myelination was first detected by LFB at p7, with no staining within the corpus callosum at p0. At p7 several myelinated axonal cross-sections were visible. By p14 the number of cross-sections had increased as well as the visibility of longitudinal LFB-sensitive axonal sections. At p21 and p28 the number of both cross- and longitudinal-myelinated axonal sections did not increase, however the strength of the staining clearly did. By p28 the structures of the brain were clearly visible.

MBP-immunohistochemistry generally showed a similar progressive pattern of increasing MBP-positive axons within the mediolateral corpus callosum (figure 6, Red). At p0 small distinct MBP-positive regions are visible along the corpus callosum, indicating myelinated axonal cross-sections. At p7 these appear to have reduced in number and size, as there is minimal MBP-staining throughout the sample. At p14 the corpus callosum is clearly visible as a heavily immunostained region, with multiple myelinated cross- and longitudinal axonal sections. The number and intensity of these MBP-positive axons continues to increase by p21 and p28, by which heavy MBP-staining is visible.

3.2 The AMIGO proteins are expressed in a similar pattern to myelin production during development

To discover whether the AMIGO proteins were localised to myelinated regions, developing mouse brains were fluorescently labelled with antibodies to AMIGO1,2 and 3 as well as MBP.

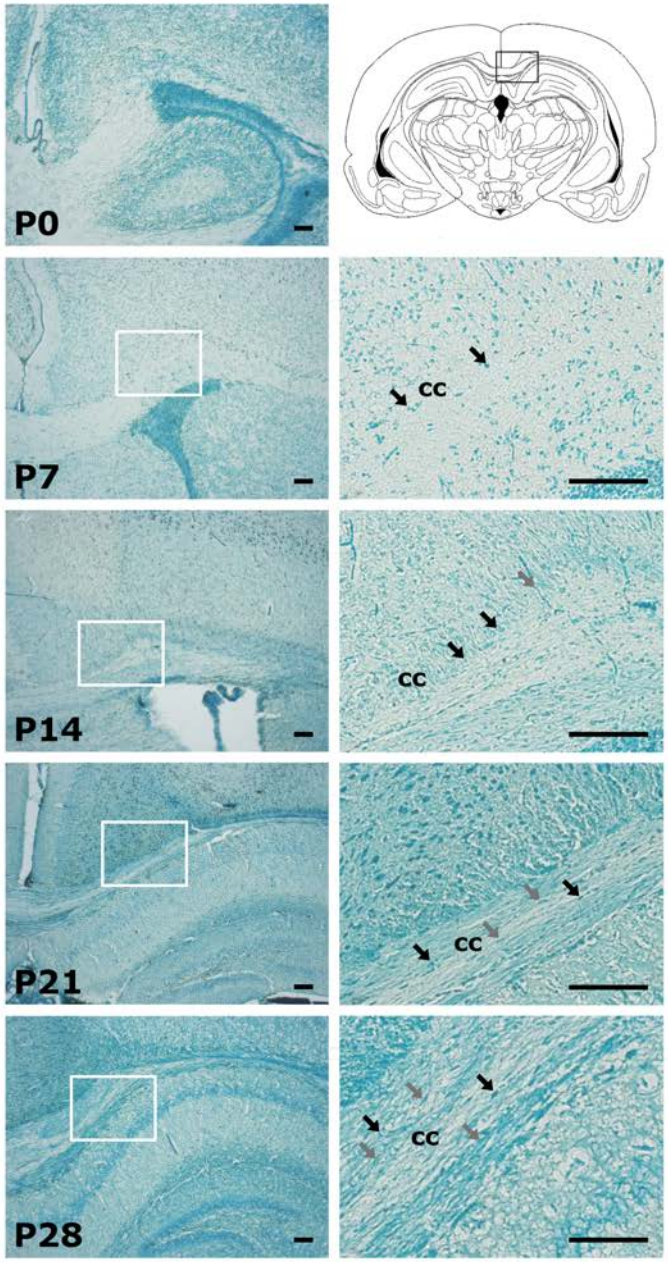

Figure 5 Progressive myelination of mouse brain from day 0 to day 28 Myelination was identified by LFB staining of the corpus callosum (cc) seen in the top right. Boxed area on left indicates higher magnification field on right. Black arrows indicate examples of myelinated axonal cross-sections. Grey arrows indicate examples of myelinated longitudinal axonal sections. Scale bars: 100µm.

Immunohistochemistry detecting AMIGO3 (figure 6, Green) showed a slightly different pattern of staining to that of MBP. At p0 there are very few AMIGO3-positive regions within the corpus callosum, with apparently fewer at p7. However, these small regions also showed strong myelination, exhibited by strong colocalisation of fluorescence (figure 6, Merge). By

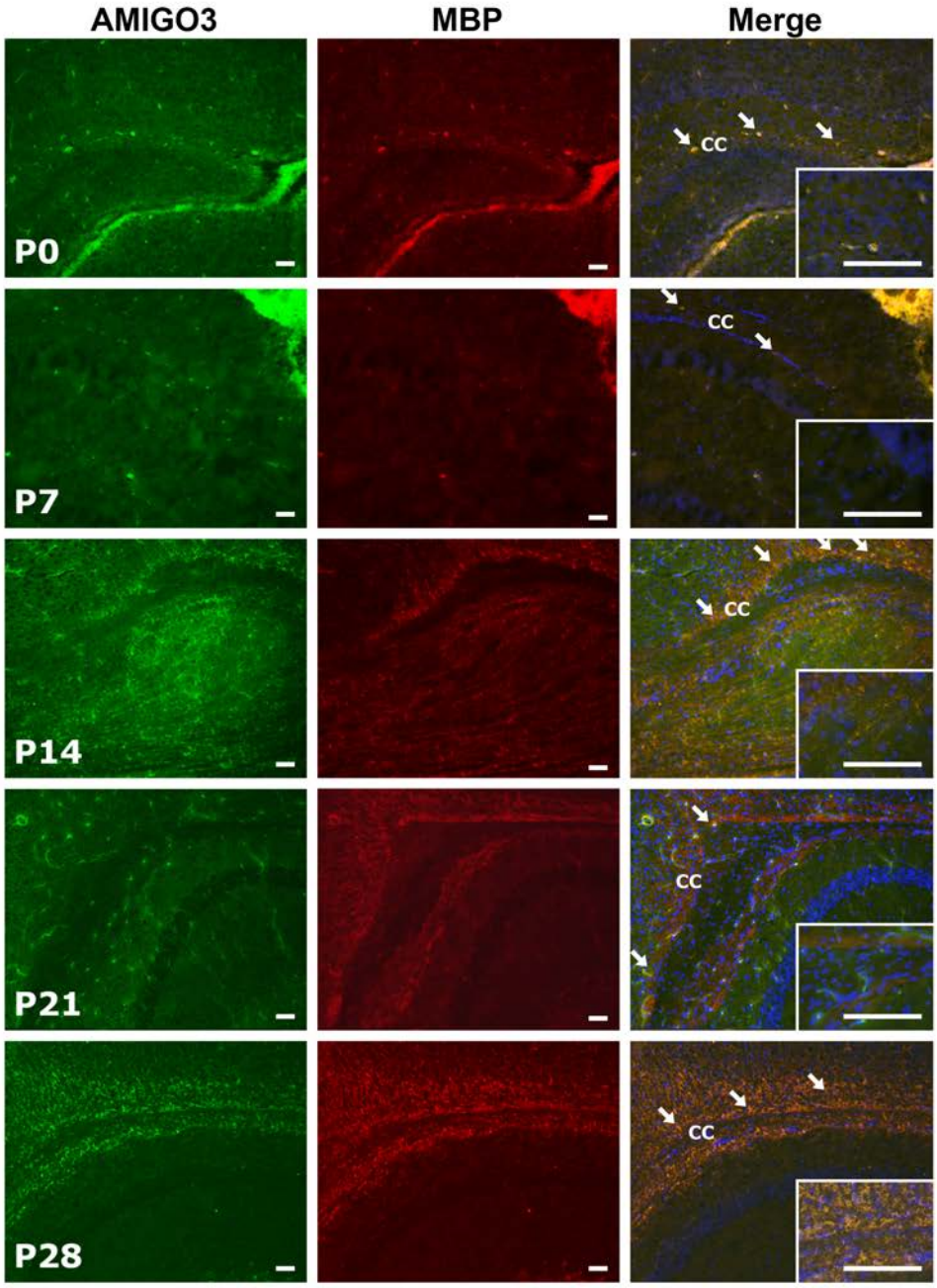

Figure 6 AMIGO3 and MBP expression in mouse brain from day 0 to day 28 MBP (red) and AMIGO3 (green) expression was highlighted by immunofluorescent staining of the corpus callosum (cc). Boxed area in bottom right indicates higher magnification field. Arrows indicate examples of colocalised fluorescence. Scale bars: 50µm.

p14, AMIGO3 presence appears uniformly increased across the whole brain section. Apparent visible levels within the partially myelinated corpus callosum were particularly elevated. Similarly, areas outside this region showed high levels of colocalisation, although AMIGO3 can be observed outside of myelinated regions. At p21 most AMIGO3 is not

expressed within myelinated regions, as expression is much less generalised than at p14, exhibiting smaller highly concentrated regions (mostly outside of the corpus callosum). At p28 AMIGO3 expression was again greatly increased within the corpus callosum, by which point is highly myelinated. MBP- and AMIGO3-staining patterns at this point appear identical. AMIGO1 and AMIGO2 (not shown) showed very similar expression patterns as AMIGO3 throughout development.

3.3 The AMIGO proteins are expressed by OPCs, OLs and astrocytes in EAE, particularly during relapse

The cellular expression of AMIGO1-3 was subsequently investigated during the process of remyelination, using the MS disease model of EAE in mice. Expression within different cell

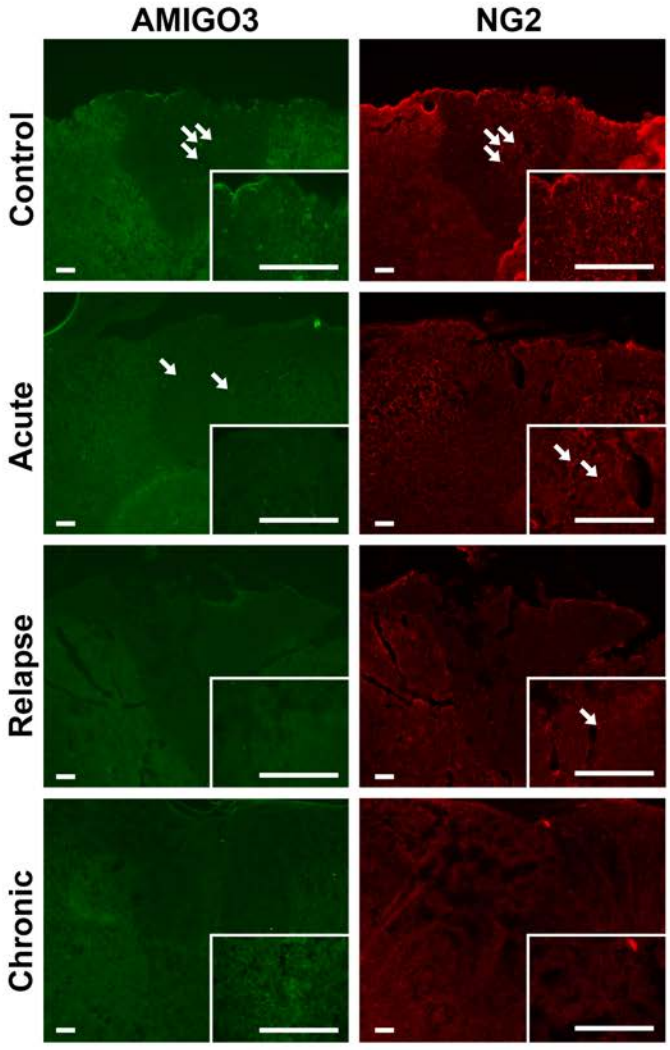

Figure 7 AMIGO3 and NG2 expression in mouse spinal cord during EAE progression NG2 (red) and AMIGO3 (green) expression shown by immunofluorescent staining of the dorsal column. Boxed area in bottom right indicates higher magnification field. Arrows indicate examples of colocalized fluorescence. Scale bars: 50µm.

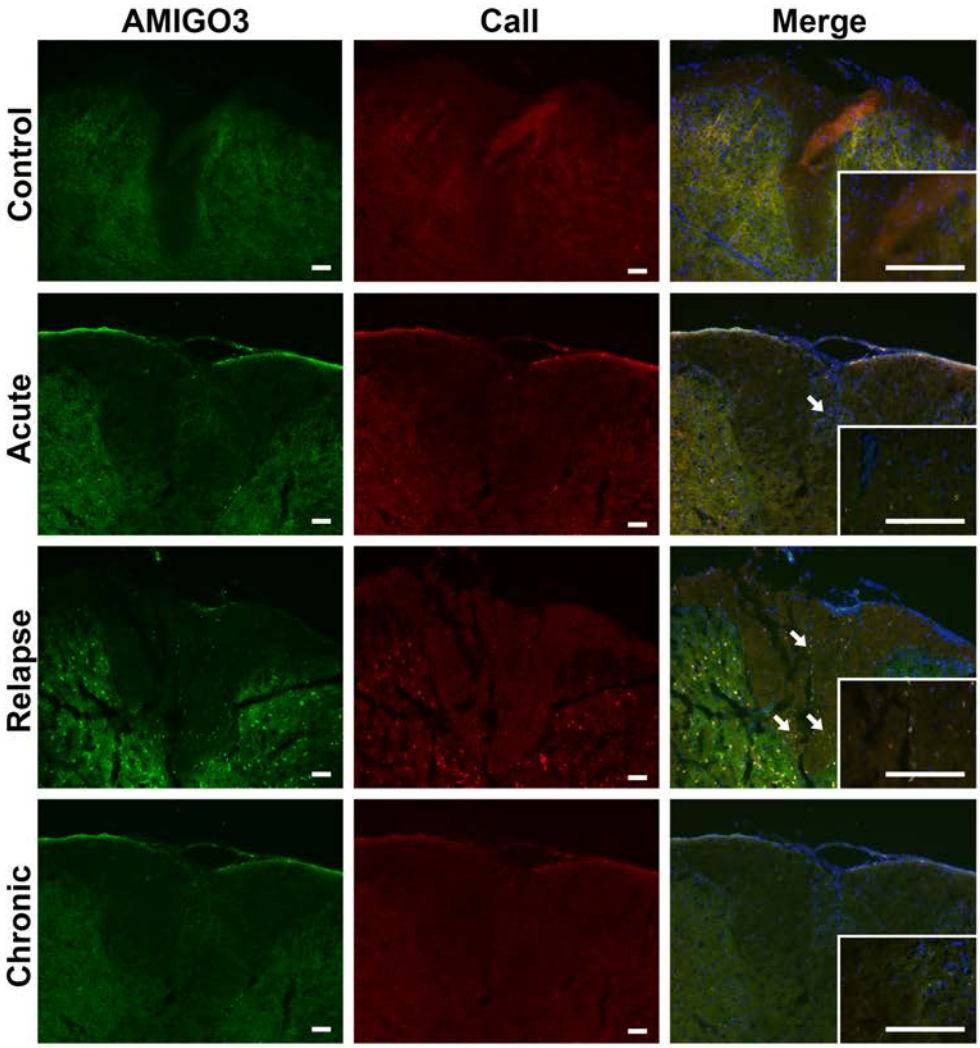

Figure 8 AMIGO3 and Call expression in mouse spinal cord during EAE progression call (red) and AMIGO3 (green) expression shown by immunofluorescent staining of the dorsal column. Boxed area in bottom right indicates higher magnification field. Arrows indicate examples of colocalized fluorescence. Scale bars: 50µm.

types was examined during the 3 stages of EAE: Acute, Relapse and Chronic. The dorsal column of the spinal cord was chosen due to the presence of the posterior column-medial lemniscus (PCML) pathway, a pathway composed of large myelinated fibers, of which a change in myelination state would be clear. The expression of AMIGO3 in OPCs was first investigated as cells that are key to myelination by immunofluorescent staining for neuron-glial antigen 2 (NG2). Clear populations

of NG2 glia are present within the dorsal column of control mice (figure 7, Red), which appeared to express AMIGO3 (Green). This was particularly noticeable at higher

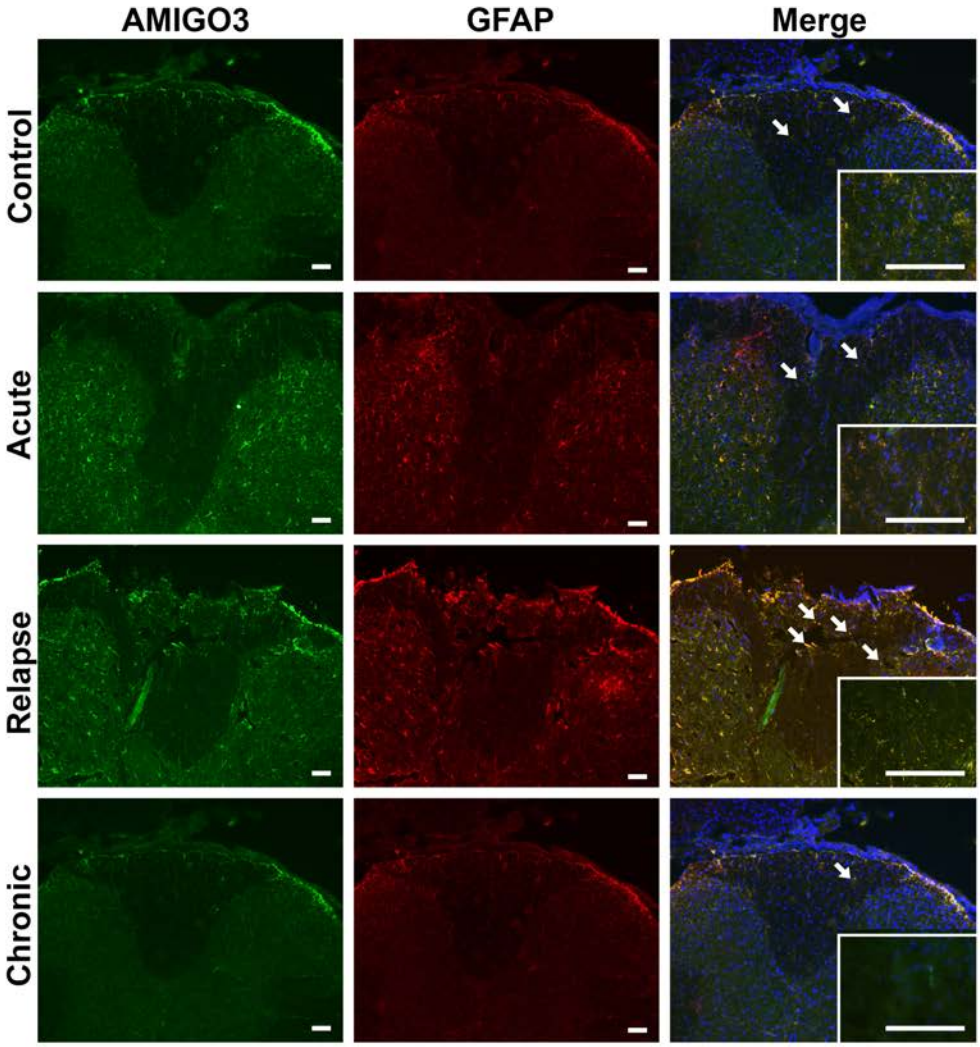

Figure 9 AMIGO3 and GFAP expression in mouse spinal cord during EAE progression GFAP (red) and AMIGO3 (green) expression shown by immunofluorescent staining of the dorsal column. Boxed area in bottom right indicates higher magnification field. Arrows indicate examples of colocalized fluorescence. Scale bars: 50µm.

magnifications. During acute stage EAE, a reduced number of NG2-positive OPCs were present, indicated by a reduction in staining, while AMIGO3 staining is further reduced indicating a decreased expression of AMIGO3. It is not possible to elucidate fully whether the AMIGO3 expression is from the OPCs during this stage. During the relapse stage of the disease there were very few, if any, OPCs present with little AMIGO3 expression. No staining was visible by the chronic stage suggesting the absence of NG2-positive OPCs. AMIGO 1 and 2 expression patterns (not shown) were very similar to those observed for AMIGO3.

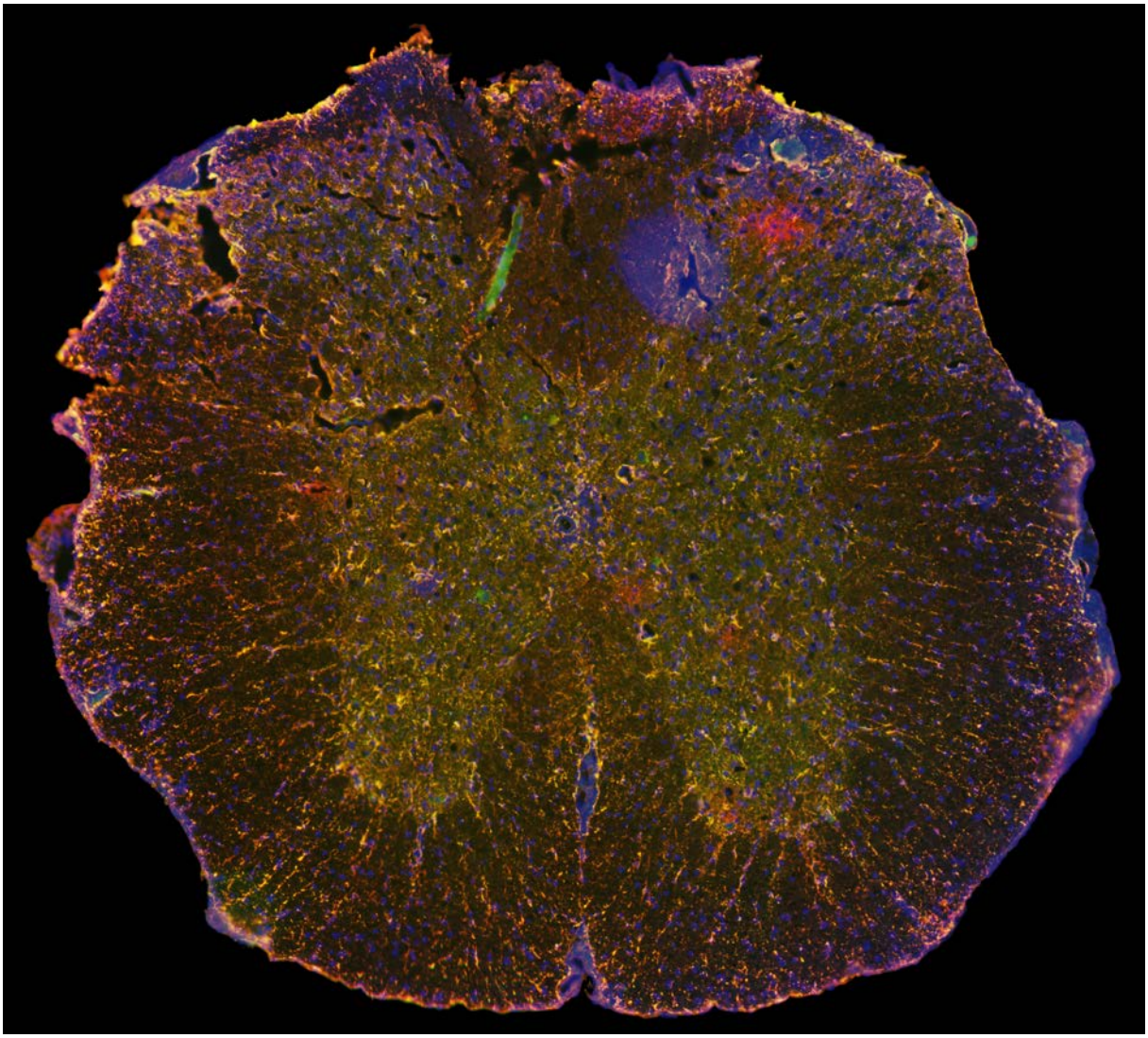

Figure 10 AMIGO3 and GFAP expression in mouse spinal cord during relapse of EAE
GFAP (red) and AMIGO3 (green) expression shown by immunofluorescent staining of the spinal cord.

A clear absence of carbonic anhydrase II (CaII)-positive mature oligodendrocytes was observed in the dorsal column of control mice (figure 8, Red) with a similar lack of AMIGO3 expression (Green). During the three disease stages of EAE all visible oligodendrocytes expressed AMIGO3 relatively strongly. During the acute stage low numbers of oligodendrocytes were observed (particularly within the dorsal column), correlating with low levels of AMIGO3 expression. During the relapse stage expression level appeared to be highest, with levels outside the dorsal column appearing higher than within. By the chronic stage there was again no presence of CaII-positive mature oligodendrocytes or any of the AMIGO proteins.

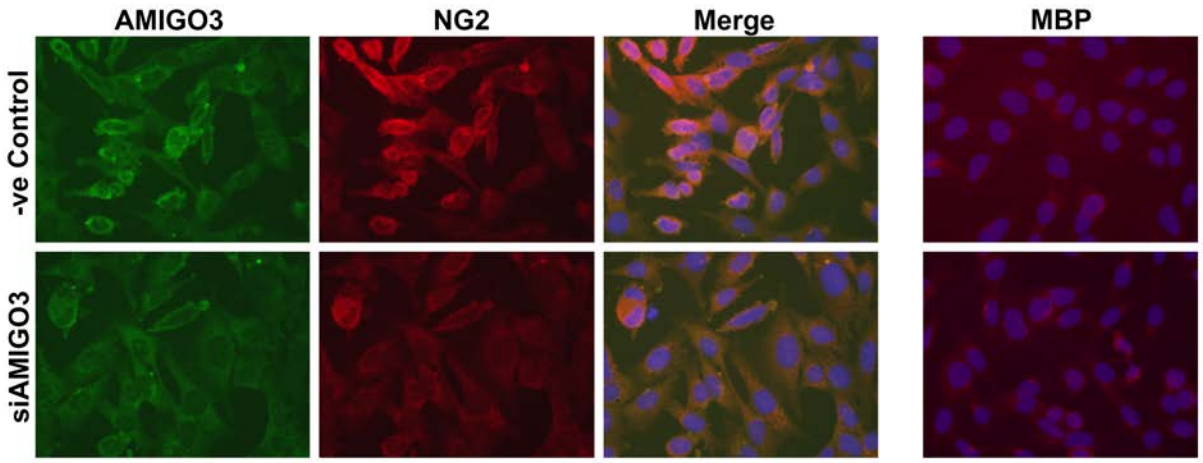

Figure 11 siRNA knockdown of AMIGO3 in the RGC5 cell line AMIGO3 (green) , NG2 (Red, centre) and MBP (Red, right) expression shown by immunofluorescent staining of the RGC5 cells.

Within control mice, low numbers of glial fibrillary acidic protein (GFAP)-positive astrocytes were present (figure 9, Red). These numbers increased considerably during the acute stage of EAE, particularly outside the dorsal column. A further increase was seen when compared to the relapse stage, during which astrocyte numbers peaked. By the chronic stage of the disease, however, these numbers dramatically decreased, although some still remained. Throughout the disease progression (and control) astrocytes expressed AMIGO3 (figure 9, Green) although during the acute stage of EAE some cells appeared to express none. The number of astrocytes, and also AMIGO3 expression, increased from control to acute to relapse and had decreased by the chronic stage. The strong expression of AMIGO3 by astrocytes was evident throughout the whole spinal cord during the relapse stage of EAE (figure 10). Similar expression patterns were observed for AMIGO1 and 2, suggesting astrocytes also produce these proteins.

3.4 AMIGO3 knockdown did not affect OPC differentiation

In order to determine the effect of reduced AMIGO3 within OPCs, particularly on differentiation, siRNA to AMIGO3 was introduced into the RGC5 cell line. Although classically considered a retinal ganglion cell line, RGC5s have recently been proposed to possess multiple OPC markers and characteristics (Ahmed, unpublished data). The presence of AMIGO3 was determined by immunofluorescent staining of cultures as well as NG2 as a

marker for OPCs and MBP as a marker for mature oligodendrocytes and myelin. AMIGO3 and NG2 expression appeared slightly reduced when compared to that of the negative control, although the effect was minimal (figure 11). There was no apparent change in MBP expression, which remained low throughout, suggesting no change in differentiation state of the cells.

4. Discussion

This study suggests that a group of LRRIG proteins, the AMIGO family, play a novel role in the myelination of neurons both during development and after disease-induced breakdown of myelin. These proteins are expressed by astrocytes, oligodendrocytes and to a degree oligodendrocyte precursor cells. However, the involvement of AMIGO3 in OPC differentiation remains unclear.

4.1. AMIGO proteins are expressed during developmental myelination

Developmental myelination is known to occur during the late stage of embryo CNS development and continue postnatally (Paus et al., 1999). This process was observed within the corpus callosum due to being an easily identifiable region of the brain that is known to be strongly myelinated. The classical LFB stain validated the use of MBP-targeted immunostaining as both showed very similar patterns of staining. The benefit of using immunostaining is that two separate antibodies can be used for proteins of interest, a technique that was utilised to show correlations between the presence of AMIGO and myelin (MBP).

AMIGO1, 2 and 3 expression was not only seen to increase over time, but the patterns of expression matched that of myelin extremely well, evident at 40x magnification. This colocalisation provides a basis for a role for these proteins in the developmental myelination process. However, at days 7 and 21 this pattern was not followed. The decrease in AMIGO expression at day 7 has also been observed in other laboratories suggesting this to be a true finding as opposed to an experimental artifact, although no reason for this was suggested (Chen et al., 2012). This suggests that the decrease at day 21, and localisation to non-myelinated regions may also be more than experimental variation. These results suggest other possible roles for the AMIGOs as well as myelination.

4.2. AMIGO proteins are expressed by cells involved in myelination in EAE

There is some discussion within the scientific community as to the validity of the recapitulation hypothesis of myelination (Fancy et al., 2011). For this reason, both developmental and remyelinating processes were observed. The mouse model of EAE was used due to it's similarities to MS in humans (Lassmann, 1983). EAE differs to MS primarily by it's instigation, however the process of demyelination, and particularly subsequent remyelination, are thought to be largely similar making it a useful tool within this thesis (Gold et al., 2006). Similar to the developmental experiments, fluorescent immunohistochemistry was used to observe AMIGO protein expression. This allowed for investigation of AMIGO expression by particular cell types by colocalising fluorescence. However it was not possible to perform costaining with the anti-NG2 and anti-AMIGO1, 2 and 3 antibodies due to interference between the two antibodies. Unfortunately this resulted in a lack of clarity in the analysis of AMIGO expression by OPCs.

During the acute stage of EAE, myelination was expected to be higher than that of the control as it is needed to replace that lost during the immune attack. This effect was expected to further increase during the relapse as more damage has occurred, meaning more myelin to replace. By the chronic stage, as with MS, remyelination was predicted to stop (Franklin, 2002). AMIGO expression observed in this study appears to also follow this pattern; expression is higher in the acute stage than control, which further increases during the relapse stage and with none apparently expressed within the chronic stage. This further suggests a role for the AMIGOs in the process of myelination.

All three AMIGO proteins were expressed by each of the cell types investigated (astrocytes, OLs and OPCs), which had only previously been shown for AMIGO1 (Chen et al., 2012). Although it is difficult to discern if the OPCs present in the spinal cord express the AMIGOs, data using the RGC5 cell line suggest these cells do express at least AMIGO3. Suprisingly, the number of OPCs observed through all stages of disease progession was low.

Considering that after destruction of myelin mature OLs are unable to produce more myelin (Keirsteadand Blakemore, 1997), OPC migration to sites of injury and differentiation into mature OLs for subsequent remyelination would be expected to increase. However,control tissues showed the highest number of OPCs, which decreased throughout disease progression. This may be due to the destruction of OPCs during the course of EAE progression or that the majority have differentiated into mature OLs in order to instigate remyelination. This would explain the decrease throughout disease progression, as more are destroyed or differentiate with increasing severity.

This is supported by an increase in AMIGO-expressing mature OLs with disease progression until at the chronic stage when none were present. This is in agreement with other findings, that the number of OLs increases at sites of remyelination and is greater than before demyelination (Prayoonwiwat and Rodriguez, 1993). Interestingly though, the numbers within the dorsal column are lower compared to those outside**,** suggesting PCML neurons may be less myelinated. The staining also shows the absence of OLs immunoreactivity within control mice, this may be due to OLs becoming quiescent and hence losing their reactive phenotype, which is then activated after disease. The presence of AMIGO1,2 and 3 in myelinating OLs is further evidence for their involvement in the myelinating process.

The expression of the AMIGOs within astrocytes is the most striking, showing high numbers of cells that all appear to express the AMIGO proteins. Replicating previous findings of AMIGO1 expression by astrocytes both in culture and in rat brain cryosections (Chen et al., 2012). The number of astrocytes follows that of OLs, increasing from control to relapse, with very few localised during the chronic stage throughout the whole spinal column. Astrocytes are important in the promotion of myelinating activity of oligodendrocytes suggesting that the AMIGOs may also play a role in the promotion of myelination, although further work is required to determine this.

The cell markers used for astrocytes, oligodendrocytes and OPCs were anti-GFAP, anti-CaII and anti-NG2, respectively, and are well known and used in research (Baumann and Pham-Dinh, 2001; Sofroniew and Vinters, 2010). Although they are considered specific, it has been suggested that astrocytes, particularly immature cells, can also express CaII (Cammer and Zhang, 1992). This may mean that $CaII^+$ $AMIGO^+$ cells may in fact be astrocytes and not mature oligodendrocytes and hence differences between these cells will need to be confirmed by morphological criteria. However, this is likely to be attributed to a very small populaton of cells, and not all of those visible in figure 8.

4.3. AMIGO3 removal has no effect on OPC differentiation

It was originally hypothesised that AMIGO3, similarly to LINGO1, may play a role in the differentiation of OPCs to OLs. The results of suppressing AMIGO3 expression in the OPC cell line showed no real difference to control. This suggests that AMIGO3 is either not involved in the differentiation of OPCs or that it in fact promotes the differentiation process. The latter would be in keeping with other findings, such as the expression of AMIGO3 by OLs and astrocytes.

siRNA knockdown is a relatively new technique, but is now widely used as it provides a simple method of removing a gene *in vitro*. However, without looking at the percentage of protein knockdown it is not possible to discern whether there is in fact no effect on OPC differentiation or whether there was a problem with the knockdown procedure. Although a protein assay was attempted, no valid results were obtained within the timeframe due to the extremely low protein yield obtained from RGC5 cells. It is possible that the RNA did not fully bind to that of AMIGO3, therefore not disabling its expression effectively. This could have been validated by Western Blot analysis or Bradford assay, although due to time constraints these were not performed. siRNA knockdowns are also generally not 100% efficient, if this was the case residual activity of AMIGO3 may have been sufficient for normal cell

functioning. The activity of AMIGO1 and 2 may also be able to offset the downregulation of AMIGO3, although their relative expression levels were not measured.

The RGC5 cell line used is also not classically an OPC line and was originally described as a rat (*Rattus norvegicus*) retinal ganglion cell line. The cells are now thought to be of mouse (*Mus musculus*) origin and express the neural cell markers βIII-tubulin, PGP9.5 and microtubule associated protein tau as well as no classical RGC markers (Van Bergen et al., 2009). Work within this lab suggests that they are in fact OPCs (unpublished data). This is supported by previous data that shows RGCs to express AMIGO proteins but not NG2 (Chen et al., 2012). However, it is still possible that RGC5 cells do not behave like OPCs *in vivo*.

4.4. Possible involvement of the AMIGOs during myelination

The data collected within this thesis clearly suggests a role for the AMIGO family of proteins in the developing, adult and diseased CNS. Further, this data suggests a novel role for the AMIGOs in myelination. However, due to the nature of the experiments conducted it is not possible to say what the exact role of each protein, or the family as a whole, may be.

The results suggest a role in promotion of myelination, rather than inhibition like LINGO1. This is shown by the highest presence of the proteins when myelination is occurring. If the latter were true, no AMIGO protein expression during development would be expected. The expression of the AMIGOs by OLs further reinforces this theory, as these are the cells that produce the myelin sheath itself. AMIGO1 expression has also been previously shown in primary rat neural cell cultures that were selectively enriched in oligodendrocytes (Chen et al., 2012).

It is possible that the AMIGO family of proteins not only act in a dissimilar way to LINGO1, but that they in fact oppose LINGO1 directly in terms of myelination. AMIGO1 and 2 have previously been shown to promote neuronal growth and survival (Chen et al., 2012; Tomio et al., 2003) whereas LINGO1 and AMIGO3, as part of the Nogo receptor complex, are known

to promote neurite growth cone collapse (Ahmed et al., 2013). It could therefore be hypothesised that the AMIGO proteins act as positive regulators of myelination by OLs where LINGO1 acts as a negative regulator (Mi et al., 2005). This is in keeping with the results observed here. LINGO1 is thought to negatively regulate OPC differentiation and myelination by a homophilic intercellular interaction (Jepson et al., 2012). AMIGO1 is known to dimerise via the LRR domain and thought to also interact intercellularly, AMIGO2 and 3 are also speculated to act in a similar manner (Kajender et al., 2011). It is possible that the AMIGO proteins therefore either disrupt the homophilic interaction of LINGO1 or that their own intercellular interactions trigger signaling in an opposing manner to LINGO1.

LINGO1 is expressed by astrocytes in the demyelinating lesions in the MS brain and thought to modulate neuron-glial interactions by interacting with Nogo-A on oligodendrocytes (Satoh et al., 2007). Similar interactions could occur between AMIGO proteins, supported by the high level of their expression in astrocytes within this study.

4.5. Future work

The AMIGO family is a remarkably understudied group of proteins considering the implications they have in development and disease. The lack of knowledge about these proteins means that there is much further research needed to understand their functioning. The results of this thesis suggest a promising novel role of the AMIGOs in myelination, however this is just a preliminary study.

Although the downregulation of AMIGO3 appeared to show no effect, it would also be of interest to extend this to the other members of the family. Further the use of a primary OPCs would be desirable to reduce any artifacts of the cell line that may adversely affect results. Considering the prediction that the AMIGO proteins may also promote rather than inhibit OPC differentiation and myelin production it would also be of interest to increase expression of the AMIGOs. Investigations with co-cultures of OPCs, astrocytes and neurons could also

yield interesting results as to whether the AMIGOs act intercellularly between different cell types and if this is necessary for function.

Blocking the function of the AMIGO proteins by administration of monoclonal antibodies during development and EAE would provide valuable information as to their role in myelination. If they do function as predicted here, by promoting myelination, the CNS may be aberrantly myelinated as well as having maldeveloped neurons. This could be investigated by further histological examination. By using antibodies for each of the AMIGO proteins individually it would also be possible to see if all had a similar effect, or to the degree in which each act. If these results were positive the next stage would be to produce a mouse knockout model of the most critical AMIGO proteins, or conditional knockout if the former proves fatal. This would allow a whole host of further examination to be carried out, from OPC differentiation to neurite outgrowth.

In order to investigate whether the AMIGO proteins also act via the same mechanistic pathway as LINGO1, binding studies, such as co-immunoprecipitation or yeast two-hybrid assays, with members of the Nogo signaling pathway could also be investigated.

4.6. Concluding remarks

Research into the role of the AMIGO family of proteins is very much in its infancy. Current work suggests members of the family are required for brain development (Zhao et al., 2011), are involved in axonal and dendrite outgrowth (Kuja-Panula et al., 2003; Chen et al., 2012) and promote survival of neurons (Ono et al., 2003). The data presented here suggests a novel role for this group of proteins as regulators of both developmental myelination and remyelination during EAE. AMIGO1, 2 and 3 expression is shown in both the developing, adult and diseased CNS of mice and their expression by several cell types involved in myelination. Reduction in AMIGO3 expression was also found to have no effect on OPC differentiation. Although preliminary, this work therefore suggests the AMIGO family as potential novel targets for therapy for demyelinating disorders such as MS.

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UNIVERSITY OF BIRMINGHAM

Investigating the roles of mesenchymal and hematopoietic stem cell conditioned media on neutrophil recruitment in a model of inflammation

by

Joseph Tickle

This project is submitted in partial fulfilment of the requirements for the award of the MRes

School of Clinical and Experimental Medicine
College of Medical and Dental Sciences
The University of Birmingham
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Abstract

Evidence suggests that both mesenchymal and hematopoietic stem cells (MSCs and HSCs, respectively) can alter an inflammatory environment *in vivo* by secretion of inflammation-modulating agents. This was examined by first developing an *in vitro* model of inflammation and subsequently adding stem cell (SC) conditioned media . Addition of basal MSC and HSC conditioned media increased Kc-activated neutrophil adhesion to TNF-α-activated colon and renal endothelium, respectively. This increase in adhesion was further enhanced when using media from SCs, which were stimulated prior to media generation. H_2O_2-treatment of MSCs and TNF-α-treatment of HSCs significantly increased neutrophil adhesion to renal endothelium compared to PBS-treated control. Similarly, when activated neutrophils were further treated with MSC conditioned media, adhesion to VCAM-1, ICAM-1, MAdCAM1 and hyaluronan increased. However, neutrophils treated with MSC conditioned media showed a decrease in adhesion to the immobilised protein ligands. Similarly H_2O_2- and TNF-α-MSC-treatment led to the significant changes in the neutrophil adhesion modulating capabilities of the supernatant. Untreated HSC conditioned media, and H_2O_2- and IFN-γ-treated HSC conditioned media treatment of neutrophils resulted in significant decreases in adhesion to immobilised protein ligands. This data suggest that MSCs and HSCs have multiple effects on inflammation.

Acknowledgements

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1. Introduction

4.1. Inflammation

Inflammation is a protective vascular tissue response to harmful stimuli, such as infection, irritation or physical damage. It is an immediate, localised response involving the immune system with the aim to remove the stimuli and initiate repair of damaged tissue. However, the process becomes dysregulated it can prove to be detrimental (Medzhitov, 2008).

The classic inflammatory response can be classified as either acute or chronic. During acute inflammation, endothelial cells are rapidly activated by inflammatory mediators, such as cytokines, which induces a range of characteristic responses. . Gaps open between endothelial cells allowing plasma proteins to pass through from the circulatory system into the damaged extravascular tissue. The matrix formed by these plasma-proteins supports the entry of leukocytes, primarily neutrophils (Pober and Sessa, 2007).

The mechanism by which neutrophils are recruited from the blood flow in order to cross the endothelium and enter the inflamed tissue occurs via the neutrophil adhesion cascade., shown in figure 1. This process consists of four defined stages, although others have been proposed (Ley et al., 2007), namely rolling, activation, firm adhesion and migration (Butcher, 1991). Each distinct stage requires different cell adhesion molecules (CAMs) in order to facilitate successful recruitment. Initially, neutrophils roll along the vessel wall, slowing their speed relative to blood flow and facilitating rolling. This stage is mainly reliant on selectins: a group of Ca^{2+}-dependent molecules present on the surface of activated vascular endothelium and the circulating leukocytes. Once slowed from flow, inflammatory cells roll along the endothelium and interact with the inflamed endothelium via transient selectin bonds to glycosylated ligands such as P-selectin glycoprotein ligand 1 (Ley *et al.*, 2007). These interactions have extremely high on- and off-rates, allowing the cells to adhere to the endothelium from flow. Subsequently, cytokines released from the endothelium activate

leukocytes.. This process is particularly rapid and can occur in under a minute (Peled *et al.*, 1999). Activated neutrophils and leukocytes upregulate adhesion molecules known as integrins, which facilitate their firm adherence to the endothelium. The integrins bind specifically to CAMs expressed by the endothelium, which belong to the immunoglobulin superfamily (IgSF). The key CAMs for firm adhesion are vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Barclay, 2003). The glycosaminoglycan hyaluronan (HA) has been shown to play an important role in leukocyte recruitment, although this is not a member of the IgSF (Kessler *et al.*, 2008). Following firm adhesion, leukocytes transmigrate through the endothelial layer, then through the basement membrane and ultimately into inflamed tissue.

Recruited neutrophils are further activated within the damaged tissue by either coming into direct contact with a pathogen (if caused by infection) or by the actions of locally produced cytokines by tissue-resident cells (Medzhitov, 2008). The activated neutrophils then clear any invading organisms or damaged cells by releasing the toxic contents of their granules, such

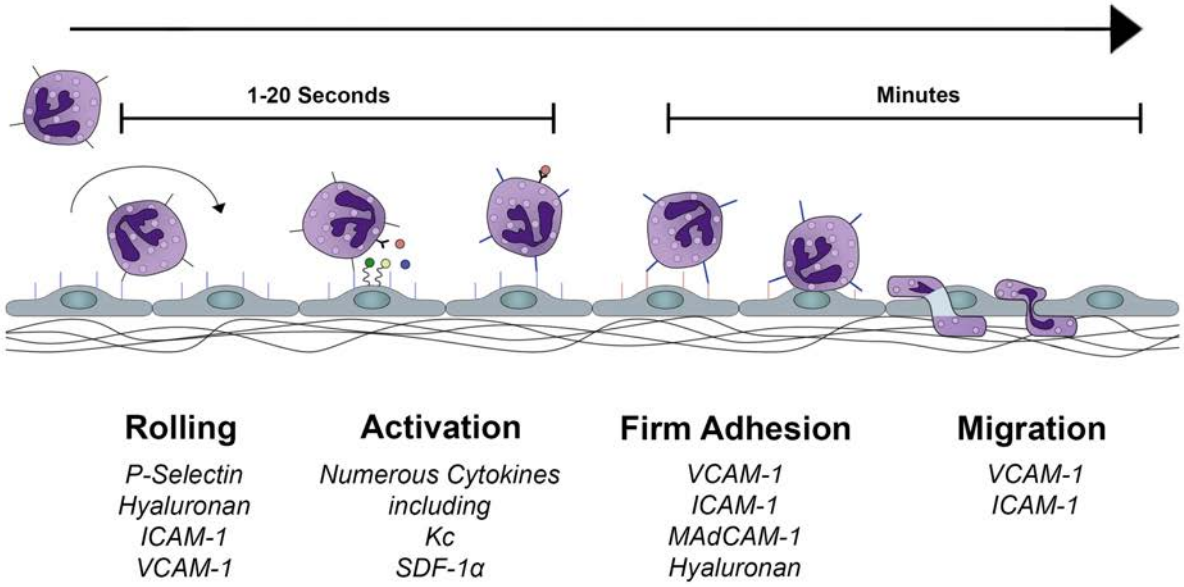

Figure 1 Leukocyte adhesion cascade The four main stages of leukocyte adhesion during inflammation are shown with illustrative examples of the CAMs and agents involved. Adapted from Butcher and Picker, 1996.

as NADPH oxidase, which generate reactive oxygen species (ROS). This process is indiscriminative resulting in destruction of healthy host tissue as well as the harmful stimuli (Nathan, 2002). Acute inflammation resolution is triggered by the neutrophils a few hours after initiation of inflammation. Neutrophils promote a change in local environment from prostaglandins to lipoxins while resolvins and protectins are also synthesised, initiating the termination sequence and preventing further neutrophil recruitment and engaging apoptosis (Serhan and Savill, 2005). The neutrophils are subsequently cleared by macrophage phagocytosis while anti-inflammatory and reparatory cytokines are released, finally followed by macrophage clearance through the lymphatic system (Bellingan *et al.*, 1997).

If the acute inflammation is not resolved, a chronic state of inflammation may ensue. In fact some pathologies tend to cause a chronic, rather than acute, inflammatory state by their nature. A non-resolving inflammatory process results in a switch from a primarily neutrophil driven inflammation to a macrophage/lymphocyte driven inflammation (particularly T-cells). T-cells are known to activate macrophages, which is a particular problem in autoimmune disease. The phagocytosing macrophages also secrete inflammatory paracrines and growth factors that may result in the formation of granulomas.

1.1 Renal and Colonic inflammation

Inflammatory disorders are known to affect almost all tissue types, including both the kidney and the colon. Renal inflammation, or nephritis, is the number 5 cause of renal failure in the US (Kidney Trust, 2013). Nephritis is the activation of the renal endothelium, resulting in the proliferation and accumulation of inflammatory cells leading to disturbance of the glomerular structure. This can be either inflammation of the glomeruli (glomerulinephritis) or of the region between tubules (interstitial nephritis). Disruption of the kidney can result in the accumulation of urea and other nitrogen-containing substances in the blood as well as proteinuria (the presence of increased serum proteins in the urine) both of which can have serious consequences including stroke and renal failure (Haroldsson *et al.*, 2008). Two of the

most common causative agents in establishing nephritis are infection and autoimmune response. Regardless of causative mechanism, the characteristics of nephritis are broadly similar, although severity differs. Many cases of nephritis resolve spontaneously, however many will follow full disease progression from acute to chronic inflammation. In many cases this will result in irreversible damage and renal failure. Treatment aids many patients before this stage, yet nephritis and nephropathy still accounts for 1.19% of total deaths worldwide (WHO, 2004) suggesting a need for further treatment options.

Inflammation of the colon is one of the primary causes of inflammatory bowel disease (IBD) and is known to affect around 1.4 million Americans, and similarly to other Western countries, that figure is rising (Abraham and Cho, 2009). Two of the main causes of IBD, Crohn's disease and ulcerative colitis, are classified as idiopathic (no specific cause is known). One theory proposes that genetic susceptibility leads to an inappropriate inflammatory response to bacteria in the gut in these chronic disorders (Abraham and Cho, 2009). As no definitive cause is known, treatments range from symptomatic agents to anti-inflammatory treatments. Anti-inflammatory agents commonly used include corticosteroids and cytokine neutralising antibodies such as infliximab. Many of these therapeutic agents remain inadequate, can cause undesirable side effects and must be used for prolonged periods, suggesting a need for new treatments.

1.2 Stem cells

Stem cells (SCs) have a unique ability self-renew as well as differentiate into a multitude of other specialised cell types (potency), as well as other SCs. Mammalian SCs can be classified as embryonic, hailing from blastocysts, or adult, which are found throughout a variety of tissues. Since the discovery that some cell types can produce others in the mid 1800s, there have been many ideas and much controversy over SCs. Bone marrow (BM) transplants, normally consisting of the allogenic transplantation of adult stem cells (ASCs) into recipients, have been used since the mid 1900s for treatment of immunodeficiency

disorders. Embryonic SCs, however, are a more recent discovery and have only been used since their isolation in 1998. In more recent times, our knowledge of SC biology has advanced greatly, but there still exists many conflicting views of how they function and the potential therapeutic uses may be.

The regenerative capacity of stem cells to repair damaged or lost tissues by differentiation is a very attractive property for therapy. Hematopoietic stem cell (HSC) transplantation by BM transfer is still widely used to treat leukemia and lymphoma patients to replace their dysfunctional immune cells that have been destroyed by cytotoxic chemotherapeutic agents or radiation therapy. This treatment is advantageous as there is no need for expansion of cells in culture prior to use and also no need to reconstitute a multicellular tissue prior to transplant. Research in the field of SCs is advancing to provide autologous grafts for treatment of wounds such as burns (Green, 2008) and damaged corneas (De Luca *et al.*, 2006). Use of *ex vivo* autologous SCs, although requiring expansion, offers potential treatment without fear of rejection. Gene therapy has also been used within the clinic combined with SCs in order to produce modified cells in which a defective gene has been corrected (Watt and Driskell, 2010). SC research also appears positive for treatment of many disorders, such as cardiomyopathy (Jakob and Landmesser, 2013), osteoporosis (Jazedje *et al.*, 2012), and autoimmune diseases (Jakob and Landmesser, 2013).

In many damaged tissues, however, engraftment of *ex vivo* isolated/expanded SCs is not possible. However, stem cells have been proposed to naturally mobilise from their niches to sites of injury or tissue regeneration (Kavanagh and Kalia, 2011). This process has been shown in a wide variety of injuries, from amputation (Guedelhoefer and Alvarado, 2012) to tissues damaged through degeneration, ischemia and inflammation (Kavanagh and Kalia, 2011). If this process can be harnessed then external SCs may not need to be administered, and a patients own SCs may be mobilised to the site of injury.

Recently, a large interest has been taken in the other properties of SCs since they have been suggested to produce antibacterial defences through the secretion of antimicrobial peptides (Krasnodembskaya *et al.*, 2010) as well as exhibiting anti-inflammatory effects after injury (Tögel et al, 2005; Kavanagh *et al.*, 2013). Recent advancements in SC function in injury have concentrated on two types of SCs; mesenchymal stem cells (MSCs) and HSCs.

1.3 Mesenchymal and hematopoietic stem cells

The majority of ASCs used in research are sourced from BM. Within the BM exists two main subsets of stem cells: mesenchymal and hematopoietic. Mesenchymal stem cells (MSCs) are a self-renewing multipotent subset of stromal stem cells that differentiate primarily into cells of the mesodermic lineage: osteoblasts, chondrocytes and adipocytes (Ucelli *et al.*, 2008). They are defined as cells that have fibroblast-like morphology that proliferate *in vitro* as plastic-adherent colonies and express CD105, CD73 and CD90 while lacking expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules (Dominici *et al.*, 2006). Although BM-derived cells that are plastic-adherent are widely used in research as mesenchymal stem cells, not all these isolates necessarily meet specific SC criteria but are still referred to under the acronym MSC, for multipotent mesenchymal stromal cell (Horwitz et al, 2005). MSCs have also been sourced from a large number of other tissues with generally similar gene expression patterns, however slight differences are exhibited (Otto and Wright, 2011).

As well as cells of the mesoderm lineage, MSCs have been recorded to transdifferentiate into cells of the ectodermic (Peterson *et al.*, 1999) and neuroendodermic lineages (Pittenger *et al.*, 1999), such as epithelial, muscle and even neuronal cells. It has been suggested that MSCs could be used for regeneration of almost any tissue, however results are often inconsistent (Uccelli *et al.*, 2008).

Since the discovery that MSCs suppressed T-cell proliferation (Di Nicola *et al.*, 2002) the majority of MSC-related research has investigated their broad range of immunomodulatory activities. *In vitro* MSCs have been shown to inhibit T-cell proliferation and production of pro-inflammatory cytokines (Di Nicola *et al.*, 2002) as well as inhibiting the generation and function of dendritic cells (Nauta *et al.*, 2008) and inhibiting antibody production by B cells (Comoli et al, 2008). On the other hand, some groups have shown MSCs to have opposite effects, thus promoting T-cell survival (Benvenuto *et al.*, 2007) as well as stimulating their activation and proliferation independently of cell contact (Crop *et al.*, 2010). This contradiction of ideas shows a clear need for further research into the immunomodulatory properties of MSCs, especially before use as in the clinic.

The other main group of BM-derived adult stem cells are HSCs, which constitute around one in every thousand bone marrow cells (Schroeder, 2010). HSCs are also self-renewing BM-derived stem cells, which primarily differentiate into a variety of blood cells of both the myeloid and lymphoid lineages. There are three subtypes of HSC; balanced HSCs (which repopulate white blood cells of both lineages equally), lymphoid-biased and myeloid biased (Schroeder, 2010). Each are considered normally functioning cells. HSCs are ordinarily maintained in pools within HSC niches in the bone marrow. Cells normally reside within their quiescent state until intrinsic or extrinsic signals trigger a response (Chotinantakul and Leeanansaksiri, 2012). Once triggered, stem cells are able to mobilise into the peripheral blood system.

As previously mentioned, HSCs, via BM transplantation, have classically been used to treat a wide range of haematological and immune deficiency disorders, particularly to aid recovery after bone marrow ablative cancer treatment. However, HSCs may possess some plasticity and can also transdifferentiate into other specialised cell types, including cardiomyocytes (Pozzobon *et al.*, 2010), endothelial cells (Elkhafif *et al.*, 2011) and adipocytes (Sera *et al.*, 2009). HSCs also appear to mobilise to sites of injury (Kavanagh and Kalia, 2011). For this

reason circulating HSCs have also been manipulated to repair damaged solid-organ tissue in conditions as varied as myocardial infarction (Orlic *et al.*, 2001), chronic limb ischemia (Tateishi-Yuyama *et al.*, 2002) and neurodegenerative disorders (Mezey *et al.*, 2003).

Similarly to MSCs, much research has been conducted into the immunomodulatory effects of HSCs, often with conflicting results. This may be due to the isolation techniques employed and the resulting population of hematopoietic tissue, which may contain a variety of other cells, including some MSCs along with other contaminating cells. Furthermore, a number of groups use different sources and preparations of the HSCs, which could further exacerbate differences in results (Kørbling and Estrov, 2003). Once in the blood, HSCs protected from phagocytosis by macrophages through CD47 signalling (Chao *et al.*, 2012). They are also known to be immune to pathogen infection *in vitro* (Kolb-Mäurer *et al.*, 2002). It has been suggested that HSCs are the first responders to an infection, and their differentiation is regulated by pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and type I and II interferons (IFN) (King and Goodell, 2011). Importantly, HSCs have also been shown to secrete such pro- and anti-inflammatory cytokines and growth factors, such as TNF-α, stem cell factor (SCF) and transforming growth factor-β1 (TGF-β1) (Granick *et al.*, 2012). HSCs can also control T-cell proliferation by upregulating CD274 expression (Zheng *et al.*, 2011).

1.4 Stem cell therapy in renal and colon injury

In vivo studies, particularly within injured colon, have shown conflicting results for tissue repair. MSCs have been shown to reduce ameliorate DSS-induced colitis in rats via anti-inflammatory action (Tanaka *et al.*, 2008). Similarly, DSS-induced colitis has been shown to be worse in BM-depleted rats, with at least partial rescue by the addition of MSCs (Yabana *et al.*, 2009). However, MSC some have suggested that the presence of MSC may be detrimental to tissue health. The number of tumours in a model of fibromatosis of the intestines has been shown to correlate directly with the number of immature MSCs present

(Wu *et al.*, 2010). It should be noted that it is suggested that this is not an effect mediated by MSCs alone; interactions between the endothelium and MSCs, keep MSCs in their immature state and allowing continued fibromatosis growth (Otto and Wright, 2011).

Studies examining the use of HSCs in renal injury have also shown similarly conflicting results as those found with MSCs in the colon. HSC mobilisation has been reported by some as detrimental to kidney regeneration (Tögel *et al.*, 2004) while others have suggested that HSC mobilisation can be renoprotective in acute kidney injury (Fang *et al.*, 2005). Much work has been conducted with crude isolates of BM-derived stem cells and beneficial effects attributed to MSCs, but this may in fact be the work of HSCs present in the BM suspension (Bussolati *et al.*, 2008).

Work within this laboratory has shown that HSCs can be recruited to sites of ischemia-reperfusion injury in the colon and kidney (Kavanagh *et al.*, 2012; White, PLoS ONE, in press). Once at the site of injury, HSCs are seen to dampen the inflammatory response, observed as a decrease in leukocyte adhesion (Kavanagh *et al.*, 2013; White, PLoS ONE, in press). However, in some cases, recruitment instigated by injury alone is insufficient for mediating anti-inflammatory effects of the disorder and pretreatment with cytokines or chemokines is necessary (Kavanagh *et al.*, 2013). This pretreatment does not appear to increase surface expression of adhesion molecules but does increase the surface integrin expression (Kavanagh, 2013).

1.5 Summary

Inflammatory disorders are an increasing problem within the Western world, particularly of the colon and kidney suggesting a need for newer, more effective treatments. Cellular therapy, through HSCs and MSCs, show a great deal of promise for such treatment. Although SCs are making their way into the clinic, much is still unknown about their function, which may lead to unforeseen complications and suboptimal use of this treatment modality.

Therefore there is a need for more basic studies into their inflammation-mediating behaviour and work to optimise their potential as therapeutic agents in inflammatory disorders.

1.6 Aims and hypothesis

The main hypothesis of this thesis is that MSCs and HSCs alter the inflammatory environment at sites of inflammation, and that pretreatment of these cells will alter this effect. More specifically the aims are:

- To develop an *in vitro* method for determining an alteration in inflammatory environment.
- To measure the change in inflammation caused by MSCs and HSCs with and without pretreatment.
- To discern whether any effect is due to actions between stem cells and endothelium, stem cells and leukocytes or both.

2. Materials and Methods

2.1 Neutrophil Isolation

Neutrophils were isolated from the femurs and tibias of donor C57BL/6 mice. Animals were sacrificed via cervical dislocation followed by detachment of hind limbs from the pelvis. Bones were cleaned by careful physical removal of muscle and connective tissue. The epiphysis of each bone was removed under sterile conditions and bone marrow removed by flushing through with EDTA (0.01% in PBS) using a 25G needle. Aggregations were mechanically separated by passage through a 19G needle and the resulting suspension passed through a 70µm filter. Cells were pelleted by centrifugation (300g, 5mins, room temperature (RT)) and resuspended in EDTA (0.01% in PBS) prior to treatment with 4ml of ice-cold water for 30 seconds to lyse erythrocytes. Cells were again pelleted (300g, 5mins, RT) following restoration of isotonicity with 2ml of 4x PBS and resuspended in EDTA (0.01% in PBS). A Percoll (Sigma-Aldrich, Poole, UK) gradient column was prepared of 72%, 64% and 52% (v/v) in PBS from a stock isotonic solution of Percoll (9 parts v/v Percoll to 1 part v/v 10x PBS). Cell suspension was loaded onto the column and spun for 30mins at 1500g at room temperature. A distinct band of neutrophils should form at the barrier between the 72% and 64% Percoll solutions. Cells were washed twice by centrifugation (300g, 5mins, RT) with Ca^{2+}/Mg^{2+} free PBS supplemented with 0.1% BSA before being resuspended in Ca^{2+}/Mg^{2+} containing PBS supplemented with 0.1% BSA.

2.2 MSC Isolation

MSCs were isolated as previously described (Morikawa *et al.*, 2009). Tibias and fibias were removed as above and washed thoroughly with PBS. Bones were then ground with a pestle and mortar and washed again with 5ml PBS. Bones were incubated in 20ml collagenase (0.2% in DMEM) for 1 hour at 37°C on an orbital shaker between 100-120rpm before placing into ice. All subsequent steps were also performed on ice. Suspension was passed through a 70µm filter, keeping bones in mortar. Bones were tapped to release cells before media was

removed and filtered again. 2.5ml of ice-cold HBSS media was added supplemented with FBS (2%), penicillin (50U/ml), streptomycin (50U/ml), L-Glutamine (2mM) and HEPES (10mM) and tapping repeated. Solution was passed through a 70µm filter cells pelleted (280g, 7mins, 4°C). Supernatant was discarded and cells resuspended in media remnants. Red blood cells were lysed by addition of 1ml sterile water for 1 second before addition of 1ml of 2x PBS containing FBS (4%). The resulting solution was made up to 20ml with HBSS, filtered and centrifuged (280g, 5mins, 4°C). Supernatant was discarded and pellet resuspended in 1ml HBSS. Single colour controls were prepared by addition of 4µl of cell suspension to 4 x FACS tubes containing 100µl HBSS. 1µl of the relevant antibody was added to each single colour control (Sca-1/FITC, CD45/PE, TER119/PE, CD140a/APC). . All antibodies were added to the main cell suspension (1µl for FITC- and PE-labeled antibodies and 2µl APC-labeled antibodies per mouse). Suspension was vortexed and incubated for 30mins on ice. HBSS was added (1ml to single colour tubes, 10ml to suspension), vortexed, and centrifuged (280g, 5mins, 4°C). Supernatant was discarded and main pellet resuspended in HBSS containing PI. Labelled MSCs were isolated using flow cytometry.

2.3 Cell Culture

An embryonic murine hematopoietic progenitor cell line, HPC-7, was used for all HSC experiments within this thesis (Professor Leif Carlsson, University of Umeå, Sweden). Cells were cultured in StemPro-34 Serum Free Medium (Life Technologies, Paisley, UK) supplemented with penicillin (50U/ml), streptomycin (50U/ml), L-Glutamine (2mM) and SCF (100ng/ml, Invitrogen). Cells were maintained daily at a density of 1 x 10^6 cells/ml.

Primary MSCs were cultured in αMEM (Life Technologies) supplemented with FBS (10%), penicillin (50U/ml), streptomycin (50U/ml), L-Glutamine (2mM) and TGFβ (**10ng/ml**) and incubated at 37°C with 5% CO_2.

Immortalised renal and colonic endothelial cells **were isolated from the ImmortoMouse (Jat *et al.*, 1991).** These cells were obtained as a kind gift from Dr. J. Steven Alexander (LSU-HSC, Los Angeles, USA). Endothelial cells were cultured in flasks precoated with gelatin (2%, from porcine skin, Sigma) in growth media consisting of D-valine MEM (PromoCell, Germany) supplemented with FBS (10%), penicillin (50U/ml), streptomycin (50U/ml), L-Glutamine (2mM), non-essential amino acids (**1%**), MEM vitamin solution (1%, Gibco, Invitrogen) and mouse IFNγ (10units/ml) and incubated at 33°C with 5% CO_2.

Before use, endothelial cells were transferred to 24-well plates precoated with gelatin for 5-10mins (2%, porcine skin, Sigma) in experimental media consisting of high glucose DMEM (Sigma-Aldrich) supplemented with FBS (10%), penicillin (50U/ml) and streptomycin (50U/ml) and incubated at 37°C with 5% CO_2.

2.4 Cell Counting

A standard Neubauer haemocytometer was used for all cell counting. Cell suspensions were diluted 1:1 (v/v) with Trypan blue in order to indicate dead cells that could then be excluded from the final count. Approximately 10µl of solution was viewed under a 10x objective. An average number of viable cells was calculated from four grid fields accounting for dilution and multiplied by $1x10^4$ to yield the number of cells per ml.

2.5 Conditioned Stem Cell Media Preparation

For both HSCs and MSCs, three types of conditioned media were produced: pre-treatment media (PreTM), treatment-containing media (TCM) and post-treatment media (PostTM). 6 treatments were applied to the stem cells: PBS, CXCL1 (Kc), stromal cell-derived factor-1α (SDF-1α), H_2O_2, interferon-γ (IFN-γ) or tumour necrosis factor (TNF-α).

5ml of HPC-7 in experimental media were placed in a T25 flask at a density of $1x10^6$ cells/ml. After 24 hours, the cell suspension was removed and centrifuged to pellet cells (300g, 5mins, RT). Media was removed and retained as PreTM. Cells were subsequently resuspended in

5ml of experimental media containing treatment (25ng/ml) or an appropriate amount of PBS as control. After 24 hours, the media was removed and retained as TCM media and cells were again resuspended in 5ml experimental media not containing treatment. After another 24 hour incubation period the media was collected as PostTM and cells discarded. This process was repeated three times to ensure reliability of results. All media was stored at -20°C in 100µl or 500µl aliquots for use at a later date.

Primary MSCs were **plated into a T25 flask and at confluence,** the same procedure as above was carried out. As MSCs are adherent cells, the media was removed and cells were washed with PBS at each stage.

2.6 Fluorescent Neutrophil Labelling

Cells were labeled using 5'6-carbxyfluorescein diacetate succinimidyl ester (CFDA-SE). CFDA-SE is a cell permeable dye that is intracelullarly cleaved by esterases to a fluorescent molecule, CFSE. Neutrophils were suspended in 2ml PBS (containing Ca^{2+}/Mg^{2+}) supplemented with 0.1% BSA. 2ml of PBS containing CFDA-SE (10µM) was then added to the cell suspension and incubated for 15mins at room temperature in the dark. Staining was quenched with 6ml of neutrophil media consisting of DMEM (Invitrogen) supplemented with FBS (10%), penicillin (50U/ml), streptomycin (50U/ml) and L-Glutamine (2mM). Cells were then washed twice by centrifugation in neutrophil media (300g, 7mins, RT) and recounted. Cells were diluted to $2x10^6$ per ml for pretreatment or adhesion assay.

2.7 Stimulation of Neutrophils

Neutrophils were stimulated after fluorescent labelling by one of 6 pretreatments: PBS, Kc, SDF-1, H_2O_2, IFN-γ or TNF-α. Treatments were prepared in neutrophil media at double concentration (200ng/ml) and added to cell suspensions of $2x10^6$ cells/ml (also in neutrophil media) in a ratio of 1:1 (v/v). This solution was incubated in the dark for 20mins at room temperature.

2.8 Adhesion Molecule Static Adhesion Assay

Flat bottomed 96-well plates were coated with one of four adhesion molecules: vascular cell adhesion molecule-1 (VCAM-1, 10µg/ml), intercellular adhesion molecule-1 (ICAM-1, 10µg/ml), mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1, 10µg/ml) or hyaluronic acid (HA, **0.5**mg/ml). Plates were incubated for 1h at room temperature. Adhesion molecules were then aspirated, the plates washed with PBS. Plates were then blocked with 1% heat-denatured BSA in PBS for one hour at room temperature.

1 x 10^5 neutrophils were loaded into each well and incubated for 20mins at room temperature. Where appropriate, conditioned media was also added simultaneously (100µl). After incubation, cell suspension was aspirated and washed three times with PBS carefully to avoid detachment of adherent cells by rough handling. Adherent neutrophils were then fixed by incubation with formalin solution (5%, Sigma-Aldrich) for 15mins at 37°C. Wells were washed three times with PBS before the addition of 100µl PBS. Plates were then stored at 4°C until ready for analysis.

2.9 Endothelial Cell Static Adhesion Assay

24-well plates were precoated with gelatin (2%, porcine skin, Sigma) to aid endothelial cell adhesion. This was then aspirated and washed with PBS to remove excess. Endothelial cells (either kidney or colon) were grown to confluence in experiment media at 37°C with 5% CO_2. Media was removed from the monolayers and washed with PBS. Endothelial cells were activated with TNFα (100ng/ml in fresh media) for 4 hours at 37°C. Media was then removed, monolayers washed with PBS and 5 x 10^5 fluorescently stained neutrophils activated with Kc added (in 500µl neutrophil media). Conditioned stem cell media was simultaneously added in a ratio of 1:1 (v/v) and incubated at 37°C for 30mins to allow transendothelial migration. Following this period, media was removed and cells washed three times with PBS before incubation with formalin solution (5%, Sigma-Aldrich) for 15mins at 37°C. Cells were again

washed three times with PBS before the addition of 500µl PBS. Plates were then stored at 4°C until ready for analysis.

2.10 Imaging and Analysis

All imaging was performed using an EVOS FL cell imaging system (Life Technologies, UK). 5 separate fields of view were selected for all static adhesion assays using a set pattern for consistency. An initial field was chosen in the center of either the 96- or 24-well plate as well as adjacent fields above, below and to the left and right of this initial field. The number of adherent fluorescent cells was counted using ImageJ analysis software (Wayne Rasband, NIH, US). Non-fluorescent cells were counted by hand.

3. Results

3.1. Gr-1 positive neutrophils can be isolated in sufficient number and purity from the murine bone marrow

In order to investigate inflammation effectively, it was important to know that the neutrophils could be cells isolated from BM in sufficient number and at sufficient purity. Flow cytometric analysis was used with Gr-1 as a marker of neutrophils. 75.16% of the total viable cells analysed were Gr-1-positive compared to 0.21% of the IgG control (Figure 2A and B). This was deemed acceptable evidence for the isolated cells to be used as neutrophils. On average one mouse yielded approximately 8,000 neutrophils.

3.2. Neutrophil adhesion to CAMs is not significantly altered by CFSE

Staining of the isolated neutrophils within experiments was important to permit simple quantitative analysis. In addition, we investigated whether staining cells with CFSE led to increased adhesion. To investigate this, a static adhesion assay was conducted with unactivated stained and unstained neutrophils. Although there was a slight numerical reduction, staining of neutrophils with CFSE did not significantly alter their adhesion to immobilised protein ligands (Figure 2E-H). Figure 2C and D show an illustrative example of stained and unstained neutrophils adherent to VCAM-1.

3.3. Neutrophil adhesion to CAMs is significantly increased by Kc

In order to model *in vivo* inflammatory conditions, a mechanism for activating neutrophils was required. In order to investigate this, neutrophils were incubated with a selection of cytokines, chemokines and reactive oxygen species (ROS) prior to CAM static adhesion assays (Figure 2A-D). Kc pretreatment of neutrophils led to a significant, almost three-fold increase of adhesion to VCAM-1 (normalised adherent cells: PBS: 2.66±0.62 vs Kc: 6.88±0.56, $P<0.01$; Figure 3A). Although H2O2 and IFNγ led to increases in adhesion to VCAM-1, these differences were not significant (Figure 3A). Levels of neutrophil adherence on VCAM-1

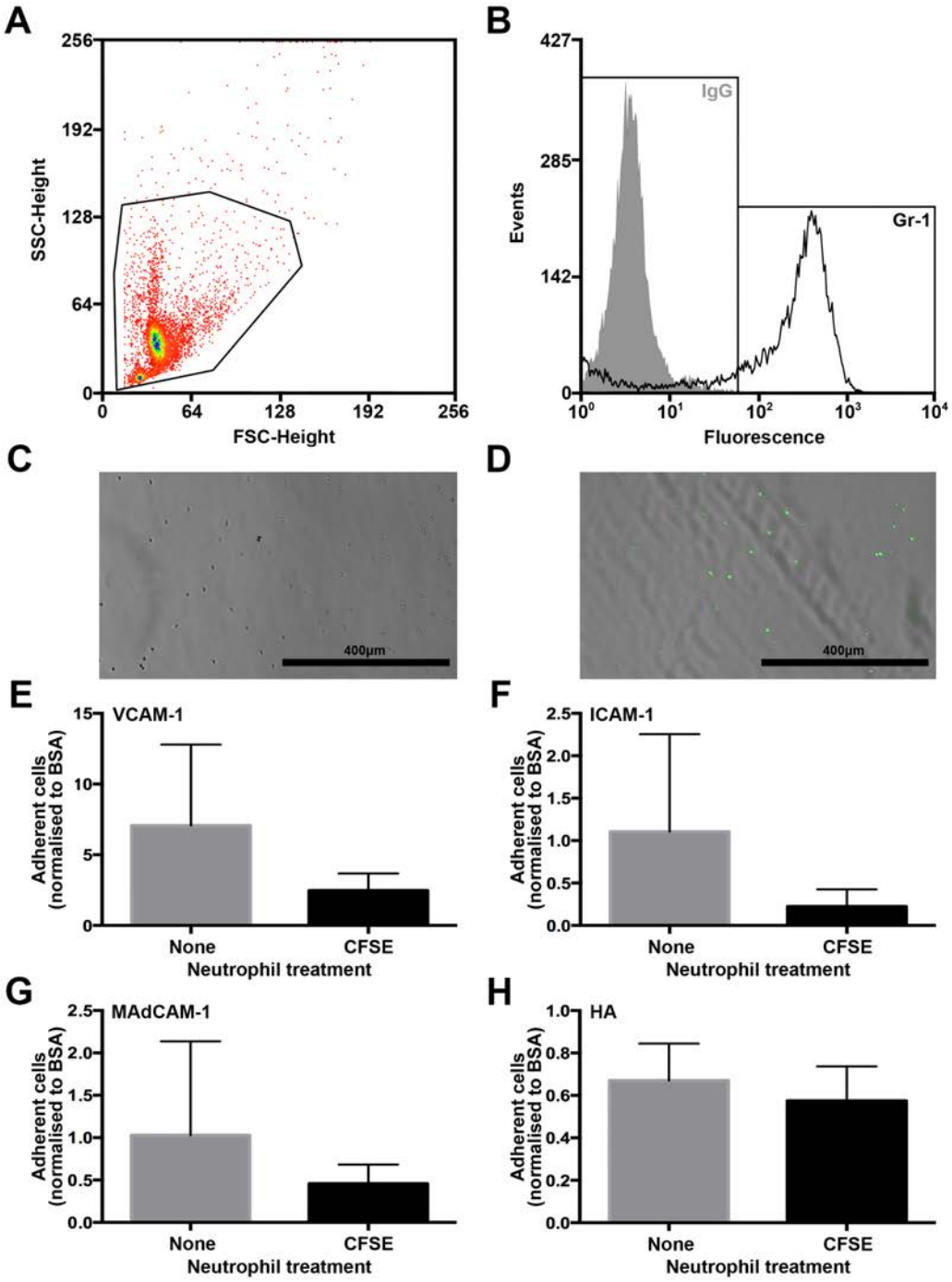

Figure 2 Flow cytometric analysis of neutrophils and neutrophil CAM adhesion assays after staining (A) Flow cytometry dot plot from a single cell suspension of BM-derived neutrophils labelled. The highlighted area shows viable cells. (B) Gr-1 flow cytometry histogram is shown from viable cells. The black line represents Gr-1 positive cell; the grey line represents the negative control (n=1). (C) Representative image of unstained neutrophils adhering to VCAM-1. (D) Representative image of stained neutrophils adhering to VCAM-1. (E-F) Number of stained and unstained neutrophils adherent to VCAM-1, ICAM-1, MAdCAM-1 and HA normalised to BSA control ± SEM. (n=4 prior to removal of statistical outliers (P>0.05)). Statistical significance was calculated by unpaired t-test, no significant difference was detected.

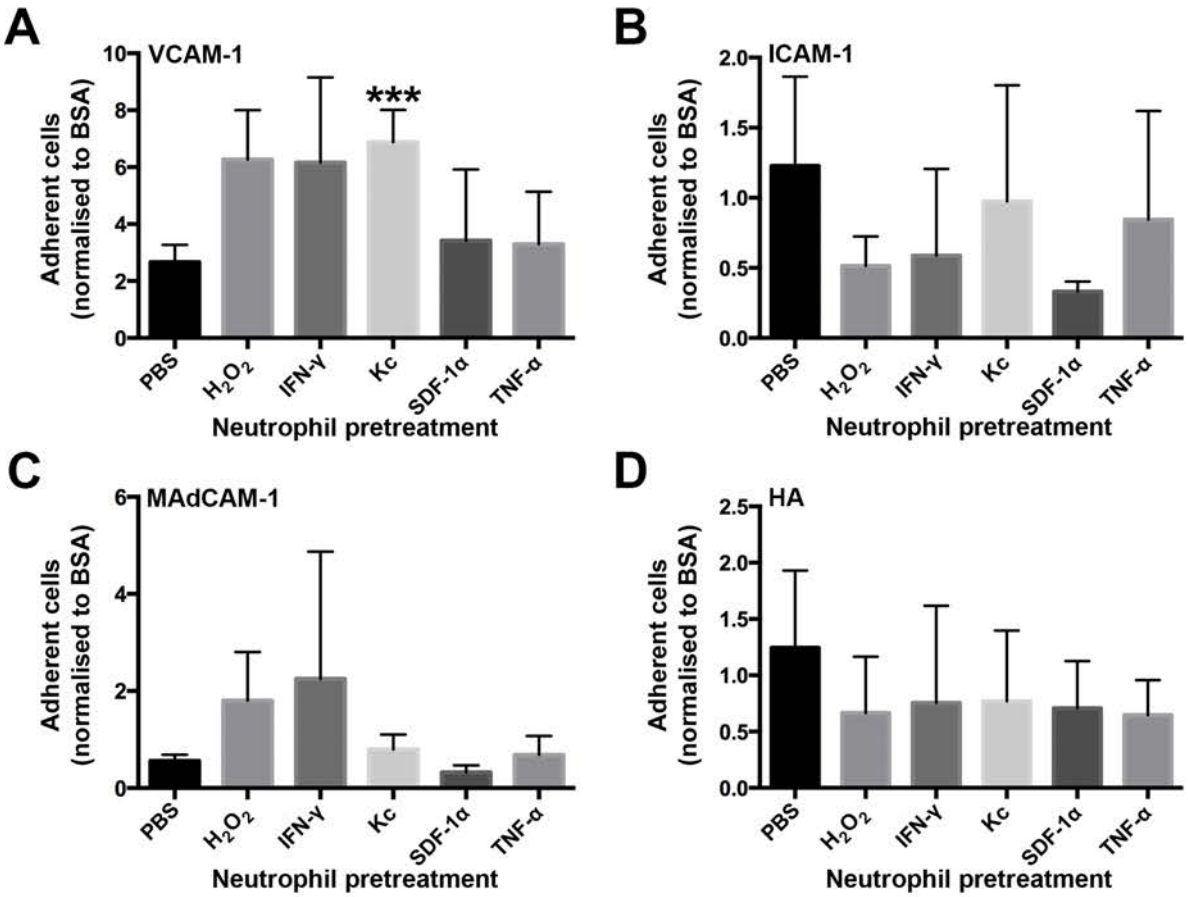

Figure 3 Neutrophil pretreatment CAM adhesion assays (A-D) Number of neutrophils adherent to VCAM-1, ICAM-1, MAdCAM-1 and HA after pretreatment, normalised to BSA control ± SEM (n=4 or 5). Statistical significance was calculated by unpaired t-test compared to PBS-treatment. ***P=0.002.

MAdCAM-1 was higher following H_2O_2 and IFN-γ treatment, these increases were not significant (Figure 3C). No pre-treatment strategy significantly altered the adhesion of neutrophils to either ICAM-1 or HA (Figures 3B and D). Kc pre-treatment was used in subsequent experiments to activate neutrophils.

3.4. Neutrophil adhesion to endothelium *in vitro* is not significantly affected by pretreatment but is proportional to the number of cells applied

During inflammation *in vivo*, both endothelial cells and neutrophils are activated. The effect of this on neutrophil adhesion was therefore investigated. Combinations of Kc-treated neutrophils were added to TNF-α-treated renal and colon endothelium (Figures 4A and B respectively). Activation of colonic endothelium or neutrophils increased neutrophil adhesion

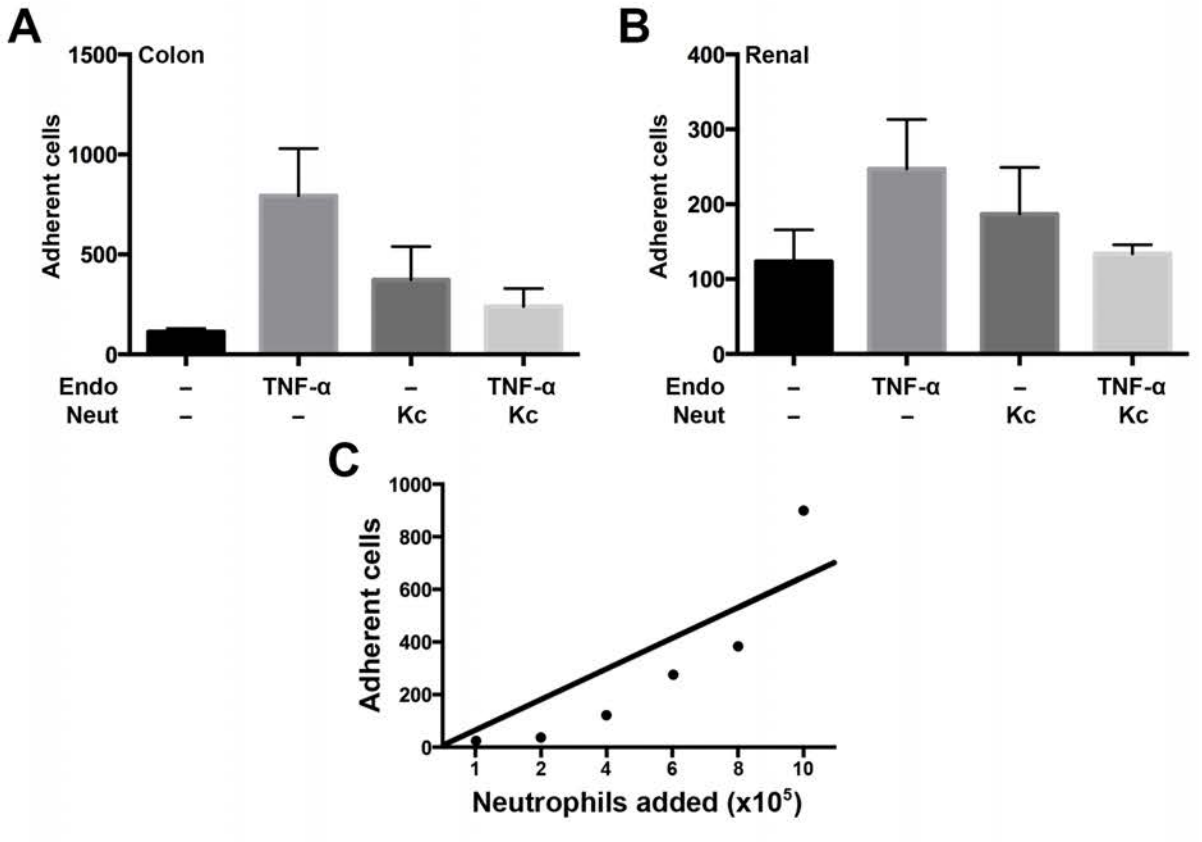

Figure 4 Neutrophil adherence to endothelium (A) Number of Kc-treated/untreated neutrophils adherent to TNF-α-treated/untreated colon endothelium (n=3). (B) Number of Kc-treated/untreated neutrophils adherent to TNF-α-treated/untreated renal endothelium ± SEM (n=4). Statistical significance was calculated by unpaired t-test compared to no treatment. No significant difference was detected. (C) Kc-treated neutrophil number titre to TNF-α-treated colon endothelium (n=1).

compared to that seen when endothelium and neutrophils were treated with PBS alone (Figure 4A). Activation of colonic endothelium alone showed the greatest increase in neutrophil adhesion, albeit insignificant (unactivated neutrophils/unactivated colon endothelium: 113.5±16.3 vs activated colon endothelium/unactivated neutrophils: 793.4±235.36, $P>0.05$) while activation of both showed only a very slight increase (unactivated neutrophils and colon endothelium: 113.5±16.3 vs activated colon endothelium and neutrophils: 239.9±89.7, $P>0.05$).

On comparison, around half the number of neutrophils adhered to renal endothelium than colon, although similar patterns in adhesion with activation were observed. Activation of renal endothelium alone also showed an increase in adhesion, however activation of both renal

endothelium and neutrophils exhibited a decrease in adhesion (unactivated neutrophils/unactivated renal endothelium: 165.8±42.6 vs activated renal endothelium /activated neutrophils: 121.8±12.04, P>0.05). None of the observed changes in adhesion were statistically significant (P>0.05). To better model *in vivo* conditions, both neutrophils and endothelium were activated in the following experiments.

A neutrophil number titre was also performed to optimise the *in vitro* inflammation assay. Between $1x10^5$ and $1x10^6$ Kc-treated cells were incubated with TNF-α-treated colon endothelium. Results showed around 0.04% of administered cells adhered to the endothelium with a corresponding increase observed with increasing cell number (Figure 4C). Subsequent experiments were performed using $5x10^5$ cells, yielding around 200 adherent cells per field of view..

3.5. Neutrophil adhesion to endothelium *in vitro* is significantly increased by presence of stem cell supernatants

The effect of pretreated MSC and HSC supernatant on inflammation was investigated by incubation with Kc-activated neutrophils and TNF-α-activated colon or renal endothelium, respectively, and observing any change in the number of adherent neutrophils. The addition of MSC PreTM to the *in vitro* inflammatory environment simultaneously with neutrophils significantly increased neutrophil adhesion compared to only the stimulated environment alone (no stem cell supernatant: 113.5±13.3 vs PreTM: 407.8±46.6, P<0.05). Illustrative examples of this increase are shown in figures 5A and B. Treatment with MSC TCMs showed no significant differences to MSC PreTM (Figure 5C). However treatment with IFN-γ-, TNF-α-, H_2O_2- and Kc-HSC PostTM showed an increase in neutrophil adhesion compared to PreTM (Figure 5D), although only the increase mediated by H_2O_2-HSC PostTM treatment was significant (PreTM: 407.8±46.6 vs H_2O_2 PostTM: 675.4±76.6, P<0.05).

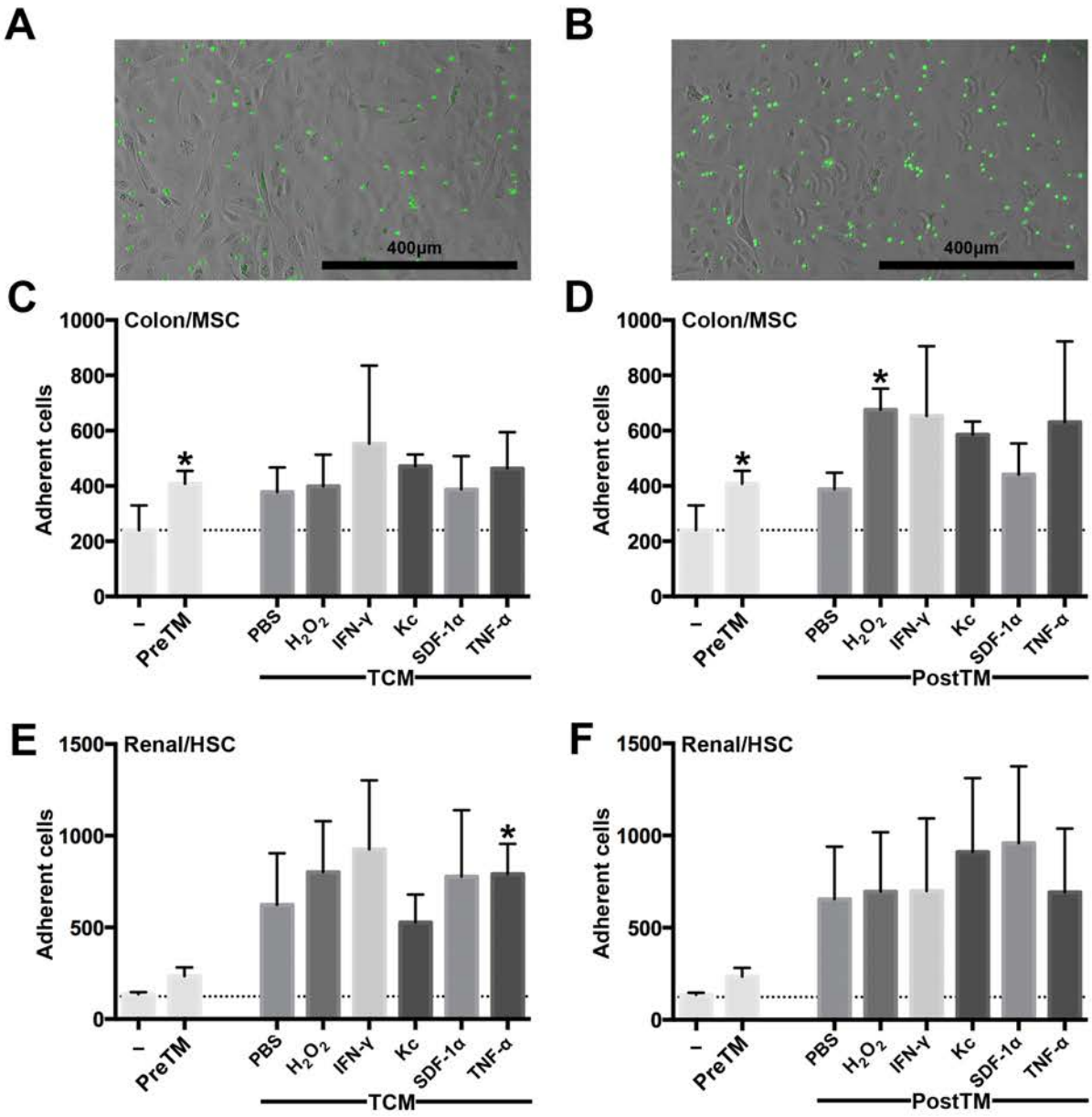

Figure 5 Effect of stem cell conditioned media on Kc-treated neutrophil adherence to TNF-α-treated endothelium (A) Representative image of neutrophils adhering to colon endothelium without stem cell supernatant. (B) Representative image of neutrophils adhering to colon endothelium in the presence of MSC-PreTM. (C-D) Number of Kc-activated neutrophils adherent to TNF-α-treated colon endothelium in the presence of MSC TCM or MSC PostTM ± SEM (n=3) (E-F) Number of Kc-activated neutrophils adherent to TNF-α-treated renal endothelium in the presence of HSC TCM or HSC PostTM ± SEM (n=3). Statistical significance was calculated by unpaired t-test. PreTM was compared to Kc-activated neutrophils alone, all other were compared to PreTM. *P<0.05.

Similarly to MSC PreTM, the addition of HSC PreTM to activated renal endothelium and neutrophils also increased neutrophil adhesion, although this was not significant (P>0.05). Incubation with all HSC TCMs increased neutrophil adhesion by between 125% (PreTM:

233.9±47.7 vs Kc TCM: 527.33±151.99, P>0.05; Figure 5E) and 296% (PreTM: 233.9±47.7 vs IFN-γ-HSC TCM: 925.4±376.6, P>0.05; Figure 5E), however TNF-α-HSC TCM exhibited the only significant difference (TNF-α TCM: 790.9±164.9 vs PreTM: 233.9±47.7, P<0.05; Figure 5E). All PostTMs increased adhesion, however, none of these groups reached significance (Figure 5F). The number of adherent neutrophils after treatment with either PBS-HSC TCM or PBS-HSC PostTM was almost identical, as expected. Other pretreatment HSC PostTMs showed no major difference to these PBS-controls.

3.6. Neutrophil adhesion to CAMs is significantly reduced by pretreatment with stem cell supernatants

CAM static adhesion assays were performed with activated neutrophils pretreated with stem cell supernatants in order to elucidate whether the previously observed increase in neutrophil adhesion upon treatment was due to an affect on the endothelium or neutrophils. Interestingly, neutrophil pretreatment exhibited the opposite effect within the CAM static adhesion assay and significantly reduced neutrophil adhesion to CAMs. Incubation with PBS-, Kc- and H_2O_2-MSC TCM post activation increased adhesion of neutrophils to VCAM-1 when compared to Kc-activated neutrophils alone although this was not significant (Figures 6A and B, P>0.05). However TNF-α-MSC TCM significantly decreased neutrophil adhesion to VCAM-1 (Kc-activated neutrophils: 6.88±0.56 vs TNF-α-MSC TCM: 2.64±1.24, P<0.05). PBS- and H_2O_2-MSC TCM also increased neutrophil adhesion on ICAM-1 whereas SDF-1α-, TNF-α- and IFN-γ-MSC TCM decreased the number of adherent neutrophils. None of these changes were statistically significant (P>0.05). MSC TCM treatments did not generally affect neutrophil adhesion to MAdCAM-1 and HA, although Kc-MSC TCM exhibited a non-significant increase (Figure 6C and D). More neutrophils adhered to MAdCAM-1 after treatment with Kc-MSC TCM (Figure 6C), as well as increasing neutrophil adhesion to HA (Figure 6D). However, both of these increases were non-significant.

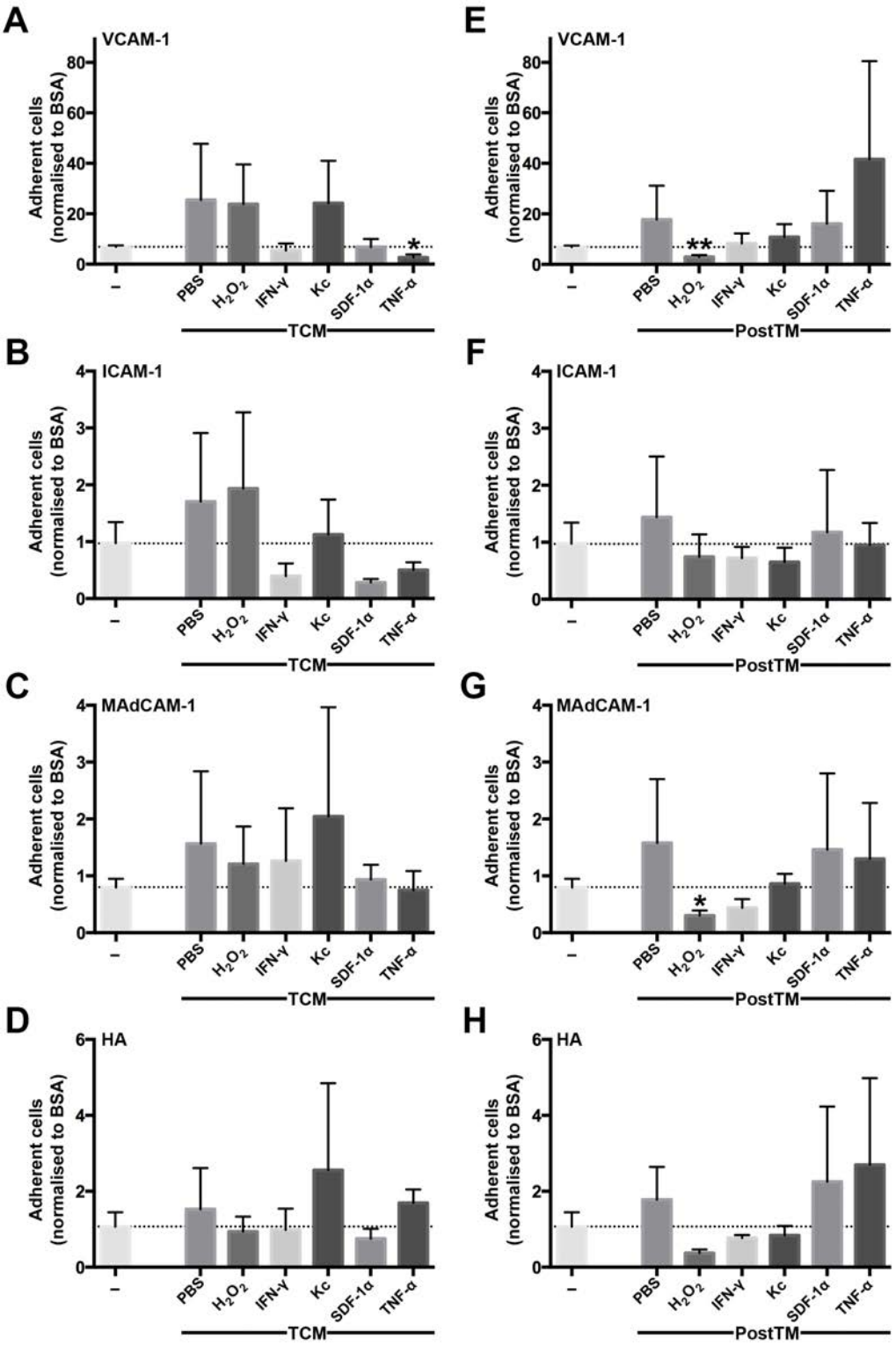

Figure 6 Kc-activated neutrophil CAM static adhesion assays after pretreatment with MSC conditioned media (A-D) Number of Kc-activated neutrophils adherent to VCAM-1, ICAM-1, MAdCAM-1 and HA after pretreatment with MSC TCM, normalised to BSA control ± SEM (n=3) (E-H) Number of Kc-activated neutrophils adherent to VCAM-1, ICAM-1, MAdCAM-1 and HA after pretreatment with MSC PostTM, normalised to BSA control ± SEM (n=3). Statistical significance was calculated by unpaired t-test compared to Kc-activated neutrophils alone. *P<0.05.

H_2O_2-MSC PostTM significantly decreased the number of adherent neutrophils compared to only Kc-activated neutrophils on VCAM-1 (Kc-activated neutrophils: 6.88±0.56 vs H_2O_2-MSC PostTM: 2.94±0.8, P<0.01; Figure 5E). All other PostTMs increased numbers of adherent cells, albeit non-significantly, by at between 20% (IFN-γ-HSC TCM) and 504% (TNF-α-MSC PostTM Figure 5E). Neutrophil adhesion to ICAM-1 remained largely similar despite MSC PostTM treatments (Figure 5F). Similarly to VCAM-1, H_2O_2-MSC PostTM significantly decreased adherency to MAdCAM-1 (Kc-activated neutrophils: 0.80±0.15 vs H_2O_2-MSC PostTM: 0.30±0.09, P<0.05; Figure 5G). SDF-1α-, PBS- and TNF-α-MSC PostTM treatment all increased, non-significantly, neutrophil adhesion to MAdCAM-1 and HA whereas the other treatments decreased it (other than Kc-MSC PostTM to MAdCAM).

Treatment of activated neutrophils with either HSC TCM or PostTM either decreased adhesion or did not affect neutrophil adhesion to VCAM-1, ICAM-1 and MAdCAM-1 (Figure 6A-H. PBS-, H_2O_2- and TNF-α-HSC TCM decreased neutrophil adhesion to around that of BSA whereas Kc-, SDF-1α- and IFN-γ-HSC TCM reduced adhesion by less of a degree (Figure 6A, n=1). Adhesion to ICAM-1 was decreased by between 16% (TNF-α-HSC TCM) and 73% (Kc-HSC TCM), although these decreases did not reach statistical significance (Figure 6B). PBS-HSC TCM treatment did not affect neutrophil adhesion to MAdCAM-1 (Figure 6C) but H_2O_2-HSC TCM and IFN-γ-HSC TCM significantly decreased neutrophil adhesion by 67% and 63% respectively to MAdCAM-1 (Kc-activated neutrophils: 0.80±0.15 vs H_2O_2-HSC TCM: 0.26±0.03 and IFN-γ-HSC TCM: 0.30±0.09, P<0.05; Figure 6C). TNF-α-HSC TCM increased adhesion to HA, albeit this increase did not reach statistical significance (Figure 6D). Although all other HSC TCMs decreased adhesion, none reached statistical significance.

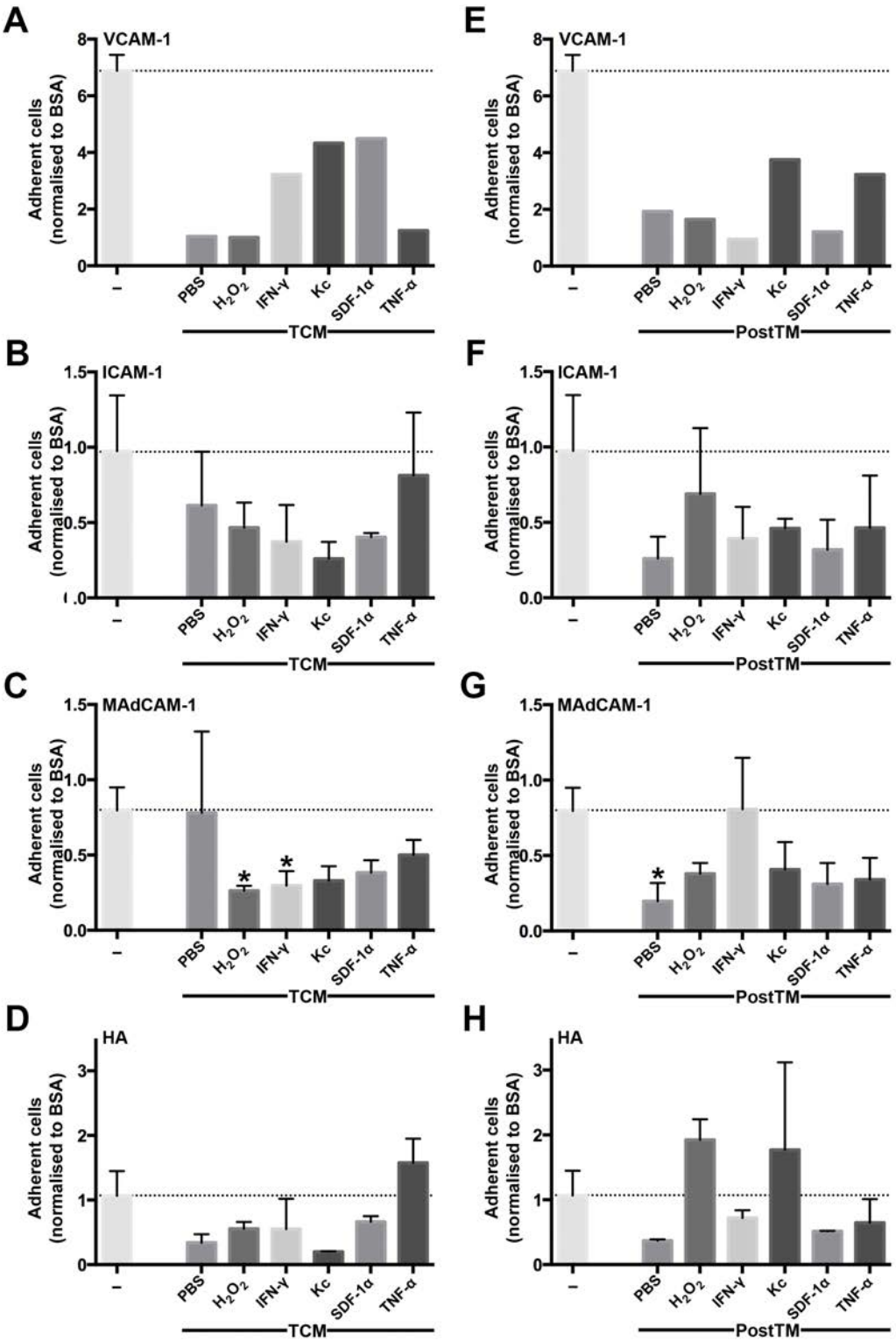

Figure 7 Kc-activated neutrophil CAM static adhesion assays after pretreatment with HSC conditioned media (A-D) Number of Kc-activated neutrophils adherent to VCAM-1, ICAM-1, MAdCAM-1 and HA after pretreatment with HSC TCM, normalised to BSA control ± SEM (n=3) (E-H) Number of Kc-activated neutrophils adherent to VCAM-1, ICAM-1, MAdCAM-1 and HA after pretreatment with HSC PostTM, normalised to BSA control ± SEM (n=3). Statistical significance was calculated by unpaired t-test compared to Kc-activated neutrophils alone. *P<0.05.

Interestingly, the greatest reductions in adhesion were seen when neutrophils were treated with PBS-HSC PostTM, with reduced adhesion seen onICAM-1, MAdCAM-1 and HA. Kc- and TNF-α-HSC PostTM decreased adhesion by 45% and 53% respectively compared to activated-neutrophils, whereas other treatments decreased adherent neutrophil numbers by between 72-86%, although these decreases were not statistically significant. All decreases in ICAM-1 adhesion were also non-significant and ranged from H_2O_2-HSC PostTM (29% reduction) to PBS-HSC PostTM (73% reduction) (Figure 6F). PBS-HSC PostTM also significantly decreased neutrophil adhesion to MAdCAM-1 (Kc-activated neutrophil: 0.80±0.15 vs PBS-HSC PostTM: 0.20±0.12, $P<0.05$). This was the only significant decrease by any PostTM treatments to any adhesion molecule. IFN-γ-HSC PostTM did not affect neutrophil adhesion to MAdCAM-1, but all others non-significantly decreased adhesion. Kc- and H_2O_2-HSC PostTMs increased neutrophil adhesion to HA non-significantly, while other PostTMs decreased adhesion by one to two thirds.

4. Discussion

4.1. Development and optimisation of an *in vitro* model to examine SC-modulated effects on inflammation

In order to investigate the inflammation-modulating activity of MSC and HSC supernatants, an *in vitro* model was developed. The assay aimed to replicate *in vivo* inflammation by measuring activated, fluorescently labelled neutrophil adhesion to activated endothelium both with and without prior treatment with previously prepared SC supernatants.

Neutrophils were isolated from murine BM by density centrifugation, which yielded a population of cells largely consisting of Gr-1-positive cells. Gr-1 expression is primarily limited to granulocytes (Fleming *et al.*, 1993), the majority of which are neutrophils. This resulted in a pool of cells consisting primarily of neutrophils but also likely to contain small numbers of other cells, including eosinophils and basophils. These cells were labelled with CFSE for ease of analysis, and we investigated cell adhesion to VCAM-1, ICAM-1, MAdCAM-1 and HA. These CAMs were chosen due to their known role in the leukocyte adhesion cascade..

In order to better model an *in vivo* environment in which neutrophils would be activated at a site of inflammation, static adhesion assays were performed to find the most suitable activator that optimally increased adhesion to CAMs. H_2O_2, IFN-γ, Kc, SDF-1α and TNF-α have all been previously shown to increase neutrophil adhesion to endothelium *in vivo*, however each promotes adhesion via a different mechanism, which may affect further experiments (Qian *et al.*, 2011; Ellis and Beaman, 2004; Zhang *et al.*, 2001; Petty *et al.*, 2009; Lauterbach *et al.*, 2008). Kc was subsequently chosen as results found it the only treatment of those screened to exhibit a significant increase in neutrophil adhesion to VCAM-1. Results were normalised to BSA due to large inter-experimental variations in the levels of cell adhesion. These variations may be due to the differences in the percentage of

neutrophils in the isolated cell populations, natural variations between animals from which they were isolated or possibly differences in preparation time. Increases seen with other treatments would may have reached statistical significance with further repeats. Kc has previously been shown to induce all stages in the extravasation process of neutrophil recruitment *in vivo* (Zhang *et al.*, 2001). Other studies have also noted a small, temporary increase in neutrophil adhesion to endothelium after Kc treatment (Bajt *et al.*, 2001) the data shown here exhibit an increase in neutrophil adhesion when treated with Kc.

Endothelium from two tissue beds were chosen for these studies, colonic and renal, due to their relation to specific inflammatory disorders *in vivo.* IBD and nephritis are inflammatory conditions of the colon and kidney, respectively, which are affecting many in the Western world. TNF-α was chosen to activate these endothelial cells due to its successful use as an endothelial activator for a long period of time in neutrophil adhesion experiments (Meekins *et al.*, 1994; Ishii *et al.*, 1994). Indeed, in this study, activation of the endothelium with TNF-α increased neutrophil adhesion to endothelium. While both TNF-α-treated endothelium and Kc-treated neutrophils displayed an increase in neutrophil adhesion to endothelium, interestingly, when treatments were combined adhesion was less than seen for each treatment individually. This is unexpected as TNF-α treatment is known to increase ICAM-1 and VCAM-1 surface expression on endothelium and we have shown here that Kc-activation of neutrophils increases VCAM-1 adhesion (Mackay *et al.*, 1994).

Three sets of conditioned SC media were generated: PreTM, TCM and PostTM. PreTM contained only constitutive secretions to examine effects of unstimulated SCs whereas TCM and PostTM were produced to examine the effects of cytokine, chemokine and ROS treatment on secretion. Relative concentrations of treatments were chosen in line with previous work (Kavanagh et al*.,* 2013;White et al*., in press,* 2013). TCM was obtained immediately following treatment so contained the stimulated secreted agents but also contained the original stimulant. Although the presence of the additional stimulant may

disguise any anti-inflammatory effects, it would be likely to contain the highest concentration of secretions. PostTM, while not containing of the original treatment agent, may contain a lower concentration of secretions, or none at all. As these supernatants were collected in succession, TCM and PostTM were also produced by a higher number of cells due to proliferation over the 48- and 72-hour period, respectively, which may also affect the comparability of the two supernatants.

4.2. MSC and HSC conditioned media exhibit pro-inflammatory effects *in vitro*

The developed inflammation assay was then used to assay the inflammatory-modulating capability of untreated SC supernatant. Work within the lab has previously shown positive effects of HSC transfer on renal inflammation and previous studies have shown MSC-colon benefits, therefore these conditions were modelled in this thesis (Van Deen *et al.*, 2013). Incubation with untreated MSC conditioned media (PreTM) exhibited a significant increase in neutrophil adhesion to colon endothelium, increasing neutrophil adhesion by 70%. Although non-significant, a similar effect was produced by HSC PreTM on renal endothelium (90% increase). These results suggest that both SCs constitutively secrete pro-inflammatory agents. MSCs have previously been shown to secrete large amounts of the pro-inflammatory cytokines IL-6 and IL-8 as well as the chemokine CCL2 (Hoogduijn *et al.*, 2010). IL-6 and IL-8 are known to increase lymphocyte adhesion to endothelium by upregulation of adhesion molecules (Watson *et al.*, 1996; Barnes *et al.*, 2011; Arndt *et al.*, 1996; Gerszten *et al.*, 1999) whereas CCL2 is thought to mediate neutrophil firm adhesion (Reichel *et al.*, 2009). MSCs are also known to basally secrete transforming growth factor-β_1 (TGF-β_1) (Di Nicola *et al.*, 2002), a cytokine that is thought to be anti-inflammatory. TGF-β_1 has been shown to decrease neutrophil adhesion to endothelium by inhibiting E-selectin and VCAM-1 expression (Gamble *et al.*, 1993; Park *et al.*, 2000). In the current study, the effect of pro-inflammatory agents must predominate that of anti-inflammatory agents.

The neutrophils used for these experiments were applied to the endothelium in the Kc-containing media used to activate them. Kc does not affect endothelial cells directly (Zhang *et al.*, 2001), however the increased activation period is likely to increase neutrophil stimulation. However, all treatments were carried out in a similar manner; therefore any exhibited effects were consistent.

4.3. MSC and HSC pretreatment with inflammatory cytokines increases the pro-inflammatory effect of conditioned media

The effect of pretreatment of SCs with cytokines and chemokines on inflammation-modulating activity was then investigated using the *in vitro* inflammation model. Interestingly, none of the MSC TCMs had any major effect on inflammation compared to PreTM despite containing the original activatory agents. However, PostTMs (other than PBS- and SDF-1α), which should not contain any of the original activating factors, exhibited a marked increase in cell recruitment. The most likely reason for this is that after 48 hours of treatment, the MSCs secrete pro-inflammatory agents at a higher rate, leading to higher concentrations in the conditioned media. H_2O_2-MSC PostTM exhibited the only significant increase in inflammation compared to PreTM (66% increase in neutrophil adhesion). TNF-α treatment of Wharton's Jelly MSCs has previously been shown to increase IL-6 expression by 25 times, which as previously mentioned increases CAM expression (Prasanna *et al.*, 2010). The results from our inflammation model also show a non-significant increase in neutrophil recruitment upon IFN-γ-MSC PostTM treatment. IFN-γ-treated MSCs have previously been shown to increase the secretion of pro-inflammatory agents, such as soluble ICAM-1, CXCL10 and CCL8 (Hoogduijn *et al.*, 2010).

Both HSC TCMs and PostTMs increased inflammation compared to PreTM, although these were mostly non-significant. Even PBS-treated HSCs exhibited a marked increase, which may be due to an increase in HSCs secreting the conditioned media. However this effect was not also seen between TCM and PostTM results. TNF-α-HSC TCM showed the only

significant increase in inflammation compared to PreTM exhibiting a 232% increase in neutrophil adhesion. Studies have identified roles for TNF-α in HSC regulation and maintenance, but no previous work has investigated the capacity for HSCs to modulate inflammation following such treatment (Pronk *et al.*, 2011; Rebel *et al.*, 1999).

4.4. Media conditioned by stimulated MSCs or HSCs exhibits anti-inflammatory effects on neutrophils *in vitro*

Static adhesion assays were performed on activated neutrophils pretreated with inflammatory cytokines and chemokines in order to elucidate whether the previously observed effects exerted by SC conditioned media were on endothelium or neutrophils. Interestingly, the results were largely different to the *in vitro* inflammation assay.

MSC TCM and PostTM exhibited differing effects on neutrophil adhesion (except for PBS treatment, which remained - as expected - largely the same). H_2O_2- and Kc-MSC TCM generally increased adhesion, whereas their relative PostTMs were anti-inflammatory. Indeed, the anti-inflammatory effects of H_2O_2-MSC PostTM on VCAM-1 and MAdCAM-1 were the only effects to reach statistical significance. The pro-inflammatory effect of the TCMs may be expected as they contain the original treatment factor. Both Kc and H_2O_2 were both previously shown to increase neutrophil adhesion to CAMs therefore their pro-inflammatory effects predominate over any anti-inflammatory effects. The opposite relationship between TCM and PostTM was observed for SDF-1α and TNF-α: the TCMs showed either no change or reduced neutrophil adhesion, while PostTMs showed no change or were enhanced neutrophil adhesion. Both SDF-1α- and TNF-α-treatment of neutrophils exhibited the lowest increase in adhesion; therefore any anti-inflammatory agents within the TCM predominated. These agents may be at the highest level in TCM and were either depleted in PostTMs, or HSCs may have subsequently secreted pro-inflammatory agents after stimulation was removed.

The potent immunosuppressive capacity of MSC secretions has been well documented but most of actions are exerted on T-cells by agents such as hepatocyte growth factor, nitric oxide and HLA-G (Hoogduijn *et al.*, 2010). However, one study in particular has shown that, when grown in co-culture with PHA-activated lymphocytes, MSCs decreased lymphocyte production of TNF-α, IFN-γ and IL-2 (Prasanna *et al.*, 2010). Pretreatment of MSCs with pro-inflammatory cytokines such as IFN-γ has also been shown to decrease inflammation in an allogenic mixed lymphocyte response model (Ryan *et al.*, 2007).

All conditioned media from treated HSCs exerted an anti-inflammatory effect on neutrophils, decreasing or not affecting adhesion to VCAM-1, ICAM-1 and MAdCAM-1 compared to none-treated. Even PBS-treatment, and therefore basal HSC secretions, markedly decreased neutrophil adhesion. The decrease exerted by PBS-HSC PostTM on MAdCAM-1 reached statistical significance, which could indicate that a large number of cells are required in order for effective concentrations of anti-inflammatory agents to accumulate, although without knowing the number of cells present it is not possible to know for sure. Interestingly, the anti-inflammatory effect predominated any pro-inflammatory effects of the treatments within the TCM. The importance of the decrease in adhesion to VCAM-1 is unclear, as the experiment requires additional replicates for statistical strength. H_2O_2-HSC TCM, despite containing the potently pro-inflammatory H_2O_2, and IFN-γ-HSC TCM showed the only significant decreases in neutrophil adhesion (to MAdCAM-1). H_2O_2-treatment of HSCs has been shown to increase their adhesion within the gut, while not affecting their proliferative ability or viability (Kavanagh *et al.*, 2012). IFN-γ is known to interact with HSCs and stimulate their activation and proliferation (Zhao *et al.*, 2010; Baldridge *et al.*, 2010). However, the effect on inflammation has not previously been investigated.

4.5. Future work

The body of literature covering the inflammation-mediating activity of stem cells is accumulating; much work is needed in order for a comprehensive understanding of their functioning. As such, the work within this thesis could be expanded further in several ways.

Previous investigations into the inflammatory-mediating activity of SCs have been conducted using coculture with endothelium (Prasanna *et al.*, 2010). This method not only eliminates any problems that may occur with freeze-thawing of supernatants, but is more relevant to *in vivo* applications. This method also could be used to investigate contact-dependent inflammation-mediating activity, whereas transwell coculture would allow investigation of the activity of secreted factors. The use of T-cell proliferation assays would also allow further insight into the more expansive view on inflammation-mediating activity.

These techniques could be further used for the optimisation of SC conditioned media to discover the optimal activation periods, treatment concentrations, possible combinations of treatments and combinations of SCs on specific endothelial types that produce the greatest immunomodulatory effects. This would be of great interest within the clinic as a therapeutic for inflammatory disorders.

The mechanism of action within this thesis has been purely speculative based on previous work, however the conditioned media could be analysed via Enzyme-linked immunosorbent assays (ELISA). The conditioned media could be screened for the presence of pro- and anti-inflammatory mediators that have been previously suggested, for example interleukins such as IL-6 and IL-8, chemokine (C-C motif) ligands such as CCL2 and CCL8, $TGF\beta_1$ and soluble CAMs. These experiments would provide mechanistic details as to how conditioned media may function, and the changes in supernatant composition induced by the treatments.

The effect of SC conditioned media could also be observed *in vivo* via intravital microscopy. The change in the number of adherent cells to inflamed organs can be monitored in this way

in real time. Treated SCs can also be administered as well as labelling internal neutrophils in order to assess *in vivo* changes in inflammation

4.6. Concluding remarks

SC therapy for inflammation is very much in its infancy and holds much promise for the future (Otto and Wright, 2011). SCs are already appearing within the clinic, however, fundamental knowledge of their effects on inflammation and the mechanisms of action have lead to criticisms their use is premature (Leone and Crea, 2006). This thesis aimed to better understand the inflammation-mediating activities of both MSCs and HSCs by *in vitro* methods. It is reported here that SC conditioned media has multiple effects on inflammation. Further, SCs in an inflammatory environment were shown to enhance the inflammation-mediating effect of the conditioned media they produced. This study not only gives a greater understanding of SC activity, but the data within could be used to inform studies for use in the clinic.

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