

The effect of sulforaphane on leukocyte-endothelial interactions and platelet activation during inflammation and thrombosis

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Abstract

Inflammation is thought to play a driving role in an expanding number of vascular and cerebral pathologies. Sulforaphane (SFN) is a naturally occurring isothiocyanate which has previously been shown to harbour anti-inflammatory properties in several immune cell types. In this work, intra vital microscopy was used to visualise the cellular interactions within the cerebral microvasculature stimulated downstream of an inflammatory challenge, namely intraperitoneal injection of LPS (0.1-4mg/kg). This resulted in a time, not dose, dependent stimulation of leukocyte-endothelial interactions; the number of rolling cells significantly increased, cells exhibited a slow rolling velocity and the number of adherent cells significantly increased versus sham or vehicle treated animals. SFN pre-treatment at 50mg/kg 24hr prior to LPS challenge (0.5mg/kg), increased the number of cells rolling through the cerebral microvasculature, significantly increased the speed at which they were rolling versus LPS treated animals and decreased the number of cells that were adherent to the pial vessel walls. These alterations were not associated with alterations in systemic cytokine levels (IL12p70, IL-6, TNF α , IL-10, IFN γ , MCP-1) or nuclear Nrf2 expression within the cerebral cortex. LPS (1 μ g/ml) increased IL-1 β release from isolated human neutrophils which also showed no alteration following SFN incubation (0.1-25 μ M). Investigation into the effects of SFN revealed that SFN significantly altered the ability of platelets to aggregation in response to several agonists (thrombin, collagen and ADP), and that this response was not associated with the intracellular Ca²⁺ movement or induction of LDH release. This work highlights the ability of SFN to alter inflammatory cell interactions within the cerebral microvasculature *in vivo*, and also indicates the inhibitory effect of SFN on isolated platelet function *ex vivo*.

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List of abbreviations

ABP – activity based probe	MCP-1 – monocyte chemotactic protein
ADP – adenosine diphosphate	MI – myocardial infarction
ARE – antioxidant response element	MPO – myeloperoxidase
BACH1 – BRCA1 associated Carboxy (C)-terminal helicase	NETs – neutrophil extracellular traps
BBB – blood brain barrier	NFκB – nuclear factor kappa-light-chain-enhancer of activated B cells
BCCAO – bilateral common carotid artery occlusion	NO – nitric oxide
BM – basement membrane	NQO-1 – NAD(P)H quinone oxidoreductase
CD – cluster of differentiation	Nrf2 – Nuclear factor (erythroid-derived 2)-like 2
CNS – central nervous system	PAMPs – pathogen associated molecular patterns
CUL3 – Cullin3	PAR – proteinase activated receptor
DAMPS – danger associated molecular patterns	PBMC – peripheral blood mononuclear cell
DIC- disseminated intravascular coagulation	PECAM – platelet endothelial cell adhesion molecule
ERK – extracellular signal-related kinases	PLC – phospholipase
FCS – fetal calf serum	PPP – platelet poor plasma
FITC – fluorescein isothiocyanate	PRP – platelet rich plasma
GPVI – glycoprotein VI	PRRs – pathogen recognition receptors
HDAC – histone deacetylase	PSGL-1 – P-selectin glycoprotein ligand
HO-1 – heme oxygenase 1	RPMI – Rothwell Park Memorial Institute
ICAM – intracellular cell adhesion molecule	SFN – sulforaphane
IFNγ – interferon γ	SOCS - suppressor of cytokine signalling
IL – interleukin	STAT – signal transducer and activator of transcription
IVM – intravital microscopy	TLR – toll like receptor
JAM – junctional adhesion molecule	TNFα- tumor necrosis factor α
KEAP1 – kelch like ECH associated protein -1	tPa – tissue plasminogen activator
LBP – LPS binding protein	uPa(receptor) – urokinase (receptor)
LDH – lactate dehydrogenase	VCAM – vascular cell adhesion molecule
LPS – lipopolysaccharide	vWF – von Willebrand factor
Maf – musculoaponeurotic fibrosarcoma	
MAPK – mitogen activated protein kinase	
MCAo – middle cerebral artery occlusion	

Declaration of Originality

All the work presented in this thesis presents original data, diagrams and text conducted and constructed by the author, Scarlett Gillespie. All other externally conducted data, diagrams and/or text presented within is referenced *in situ*.

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This is for you.

Everything I do is to make you proud x

1. Inflammation

1.1. A brief overview

Inflammation is a critical response integral to our ability to survive tissue damage following either infection or sterile tissue injury. As mammals, we have developed a highly orchestrated cellular response aimed at removing the invading pathogen or damaged host cells and repairing the injured tissue. This life preserving response, termed the inflammatory response, involves an array of circulating immune and vascular cells, as well as those making up the parenchyma of the damaged tissue itself¹. Under normal conditions this response is highly controlled in both a temporal and spatial manner through an active resolution of the inflammatory response involving removal of the initial immune effectors, in part, via their ability to produce anti-inflammatory mediators². In this way, inflammation and the innate immune response provide a crucial life preserving function. However, in certain circumstances the delicate balance of the inflammatory response involving specific coordinated immune cell activation, effector function and resolution does not occur. Loss of temporal or spatial inflammatory resolution results in over activation or chronic recruitment of immune cells within the tissue, thus amplifying, rather than clearing tissue damage. It is known that this aberrant immune cell activation is present in many pathologies, both centrally and peripherally. This includes sterile inflammatory responses such as the ischaemia reperfusion injury present in myocardial infarction (MI) and stroke, and also following bacterial infection, as is the case in sepsis³⁻⁵. The main aim of this thesis was to investigate the potential anti-inflammatory effects of SFN, and how these effects were related to alterations in cellular interactions and their ability to respond to physiological stimuli.

1.2. Sulforaphane

SFN is a naturally occurring isothiocyanate, so called due to the nitrogen-carbon-sulphur ($\text{N}=\text{C}=\text{S}$) moiety to which it owes its reactivity. Sulforaphane is released via the breakdown of its precursor glucosinolate, glucoraphanin. In nature, glucoraphanin is the most abundant glucosinolate in broccoli, explaining the high concentration of SFN that can be made bioavailable following glucoraphanin hydrolysis by the enzyme, myrosinase⁶. In a healthy plant glucoraphanin and myrosinase are spatially separated, however, following the breakdown of the plant's structure, as would be the case following insect/fungal attack; as well as food preparation and mastication during human consumption of broccoli, glucoraphanin is exposed to myrosinase and the breakdown product SFN is released. SFN then acts as part of the plant's defence system.

It appears that mammals have evolved to utilise this function for themselves. As well as having genes that are responsive to the presence of SFN (discussed later, see Figure 1.1), mammals also have the ability to further maximise the release of SFN through the presence of microbiota in the gut which contain myrosinase activity⁷. This allows glucosinolates that pass through the digestive to be broken down to increase the yield of SFN which can then enter the host system. The bioavailability of SFN in broccoli has also been shown to be affected by the transportation, storage from harvesting, and method of preparation, as well as presence of gut bacteria⁸⁻¹⁰.

Pharmacokinetic studies suggest that SFN is absorbed rapidly with first order kinetics depending on the dosing regime and the form of ingestion^{7,11}. SFN metabolites are then generally excreted within 24h, however, its pharmacodynamic effects are ongoing due to cellular uptake¹¹. Teaming the knowledge that SFN is a key molecule in the defence system of plants with the fact that mammals have adapted to maximise its release, and therefore the uptake of SFN, presents an interesting question as to whether SFN can positively affect aspects of mammalian physiology.

1.2.1. Molecular effects of SFN

Indeed SFN has been shown to be absorbed across the jejunum barrier, enter the blood stream and accumulate in cells via attachment to glutathione. The mainstay of SFN's effects are currently thought to be via gene induction. Alteration of the transcription factor Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), and the homeostatic systems in place to maintain its cellular levels and location, are central to the current dogma of SFN's anti-inflammatory actions⁶. This transcription factor, along with the antioxidant response element (ARE) it binds, represents an important cellular defence mechanism against host and environment oxidants (see Figure 1.1). The importance of the Nrf2:ARE system is highlighted in its conservation across animal species, having been previously observed in zebra fish¹².

SFN has been proposed to bind several cysteine residues with Nrf2's binding domain with cellular repressor Kelch-like ECH-associated protein (KEAP1)^{13,14}. Under normal physiological conditions, Nrf2 is bound to the cytosolic actin bound protein KEAP1. This interaction brings Nrf2 into a complex with the ubiquitin ligase CUL3, leading to continual breakdown via the 26S proteasome^{15,16}. On top of this BACH1 binds the ARE, to inhibit the interaction between Nrf2 and the ARE, and therefore decrease the production of anti-oxidant genes associated with their interaction¹⁷. This system maintains a basal level of Nrf2 and the genes it activates. Absence of KEAP1 leads to increased nuclear Nrf2 levels highlighting its role in maintaining low level expression of Nrf2 and also, its spatial constriction¹⁸. Early studies noted the high expression of cysteine residues within KEAP1, suggesting it as likely candidate to sense an oxidative cellular environment, and therefore its likely role in an anti-oxidant pathway¹³.

In line with this, BACH1 has also been shown to harbour several reactive cysteine molecules, thereby also making it a target for electrophilic/pro-oxidant attack¹⁷.

The N=C=S moiety of SFN provides an electrophilic component to the molecule which enables it to interact with positive ionic centres and form covalent bonds with cellular proteins. Most commonly, these centres are thiol (–SH) groups such as those found in cysteine residues. At these sites, SFN forms reversible bonds with the –SH containing proteins, altering their conformation and/or way that they interact with their target proteins¹⁹. In the presence of an oxidative stressor or indeed SFN, bonds formed at cysteine thiol groups lead to the dissociation of Nrf2 from KEAP1 (and BACH-1 from the ARE), allowing Nrf2 to translocate from the cells cytosol into the nucleus²⁰. Once within the nucleus, Nrf2 forms a heterodimer with a small Maf protein to bind the ARE²¹.

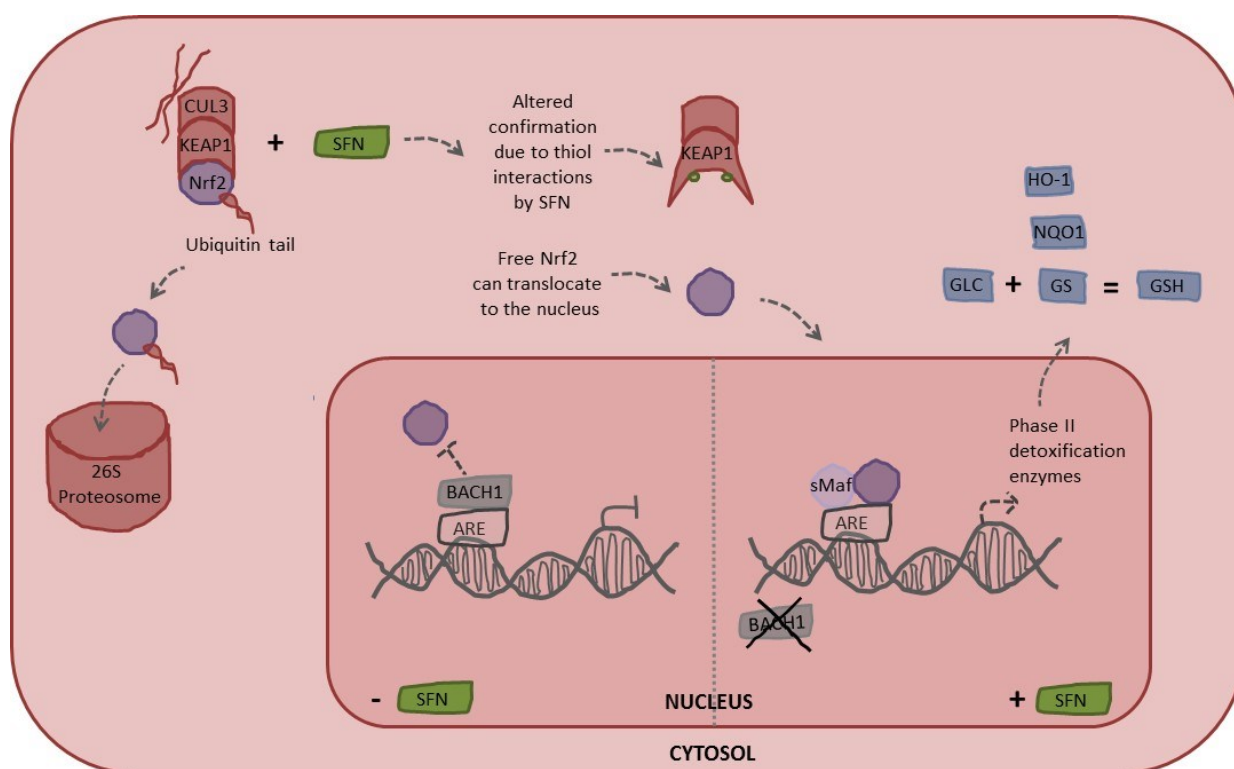


Figure 1.1: Effect of SFN on Nrf2 cellular localisation

Schematic representation of the effects of SFN on Nrf2 in a nucleated cell (Gillespie, *unpublished*)

The ARE is a 5' enhancer region found upstream of a battery of enzymes whose induction protects the cell from subsequent oxidative damage. These inducible genes can be grouped according to their function; for example, anti-oxidant regenerating enzymes, oxidant metabolising enzymes and direct anti-oxidants²². The central role that the induction of Nrf2 and the resultant production of these anti-oxidant enzymes plays has been elucidated in several inflammatory models and knock out animal models. Loss of Nrf2 results in both a reduction of basal levels of Nrf2 regulated enzymes, as well as causing an interruption of their upregulation following SFN treatment²². The fact that some enzymes are still upregulated following SFN treatment in the absence of Nrf2 does, however, suggest that SFN may act elsewhere to alter gene regulation or that these genes may also be under the control of other transcription factors. SFN's ability to induce Nrf2 regulated genes has been shown in various tissues including the vasculature, heart, lungs, liver and brain following oral, dietary, topical and intra-peritoneal administration; thus highlighting its bioavailability and pharmacodynamic ability via various routes²³⁻²⁶. The key enzymes upregulated downstream of SFN and other Nrf2 activators include HO-1, NQO-1, and enzymes involved in the maintenance of total and reduced glutathione levels. The dynamics of the induction of these genes varies between tissues and also the enzymes themselves.

1.2.2. Phase II detoxification and anti-oxidant enzymes

Heme oxygenase (HO-1): is an evolutionary conserved protein that breaks down heme to release iron, biliverdin and CO. This decreases the production of free radicals through the catabolism of heme, as well as providing anti-inflammatory effects via bilirubin and CO. Pharmacological and physiological alterations in HO-1 expression alone has been shown to alter the outcome of various inflammatory models; whereby its upregulation is associated with protection against inflammatory challenges, and reciprocally, a loss of its function or downregulation leads to increased susceptibility or worsened outcome to inflammation (reviewed by ²⁷). Induction of HO-1 by SFN in several cell studies of glial cells has been shown to peak within 24hr post treatment, and is in line with an increased survival to various inflammatory stimuli such as oxygen glucose deprivation, hemin challenge and LPS²⁸⁻³⁰. The need for the presence of Nrf2 for these effects is confirmed by the reduction in HO-1 upregulation following Nrf2 knock down using siRNA ²⁹.

NAD(P)H:quinone oxidoreductase (NQO-1) is an oxidant metabolising enzyme which aids to maintain the intracellular redox balance through elimination of 'quinone' molecules via reduction reactions, and in so doing decreases the formation of highly reactive free radical products with which these molecules are associated. Following SFN treatment, the upregulation of NQO1 mRNA and protein has been shown to be dose and time dependent in a variety of cell types; and furthermore,

this upregulation has been shown to persist longer than other Nrf2 controlled enzymes such as HO-1^{29,31,32}.

The glutathione system. The importance of the glutathione system in maintaining cellular homeostasis and protecting cellular components from oxidant damage is highlighted by the number of genes involved specifically in this system. These include: glutathione synthetase, which generates glutathione from γ -glutamylcysteine and glycine; glutamate cysteine ligase, a rate limiting enzyme composed of two subunits central to synthesis of glutathione; glutathione reductase, an enzyme vital to maintenance of the cellular pool of reduced glutathione, glutathione-s-transferase, an enzyme involved in conjugation; and glutathione peroxidase, which is involved in reduction of lipid and hydrogen peroxides³³. The fact that so many enzymes are involved in the synthesis of the glutathione system highlights the importance of its cellular regulation. Moreover, the ability of SFN to induce these enzymes in an Nrf2 dependent manner suggest the applicability of SFN to protect the cellular glutathione content and maintain the favourable balance of reduced versus oxidised glutathione in order to protect the cell²². As is seen following the knockout of other phase II detoxification enzymes, animal models lacking enzymes involved glutathione production show increased susceptibility to oxidative challenge³⁴.

Therefore, the ability of SFN to alter Nrf2, which controls the expression of these aforementioned detoxification and anti-oxidant genes presents an attractive property of the phytochemical to provide potential cellular protection against the known pro-oxidant environment to which cells are exposed to during inflammation.

1.2.3. Effects of SFN in models of pathology and innate immune cells

Of interest to this work, SFN's effects have been investigated in a plethora of both central and peripheral inflammatory models. The ability of SFN to affect, as well as cross, the BBB alone is a major finding since, the later, has previously represented a real downfall of several previous agents that have shown promise for targeting inflammatory condition within the brain^{24,35}.

In vitro findings have shown the ability of SFN to alter the inflammatory phenotype of many cells of the innate immune system including neutrophils, platelets and macrophages that are known to provide first line defence protection following infection and injury^{36,37}. Furthermore, SFN has also been shown to provide protection to cells fulfilling as barrier like function; such as endothelial cells, lining the blood vessels, and epithelial cells, covering the exterior surfaces of the body and also the gut

mucosa³⁸. Likewise, glial cells and immune effectors within the brain have also been shown to be responsive to SFN treatment^{30,39}.

In support of these data, the ability of SFN to alter whole organism responses to inflammatory pathologies has also been shown. Several pathologies known to be pre-dispose neuro-inflammatory pathologies, such as stroke, have been shown to be altered via SFN treatment; these include atherosclerosis, endothelial dysfunction and peripheral immune cell activation^{25,40}. Furthermore, the damage caused downstream of neuro-inflammatory conditions have also been shown to be ameliorated by SFN^{41,42}. This highlights the ability of SFN as a potential prophylactic treatment of 'at risk' groups both for protection against the development of key underlying co-morbidities seen in neuro-inflammatory conditions and the possible ensuing inflammation itself. Furthermore, the protection afforded in central neuropathologies indicates other beneficial effects associated SFN treatment; these include maintenance of the BBB via its tight junction, a decrease in the pro-inflammatory phenotype of activated microglial cells, protection against oedema formation and increased phase II detoxification enzyme mRNA and protein in multiple glia cells^{35,40,42}). Beneficial effects following exposure to SFN have also been shown in several epidemiological studies and early clinical trial work (See Table 1.1).

<i>Study</i>	<i>Purpose of investigation</i>	<i>Dose of SFN administered</i>	<i>Outcome</i>	<i>Significance</i>
Clarke et al, 2011 ⁴³	Pharmacodynamic study into the effects following ingestion of fresh broccoli sprouts vs. broccoli powder pill devoid of myrosinase	Sprouts: 40 g broccoli sprouts which was equivalent to 150µmol glucoraphanin Pill: 6 BroccoMax® pill which was equivalent to approx. 3 g of freeze dried broccoli sprouts and contained 121 µmol glucoraphanin	SFN metabolites post sprout ingestion peaked within 3hr and were cleared by 24hr. Following BroccoMax ingestion plasma levels peaked at 6hr and were significantly lower than those seen with sprout ingestion. Outcome was not affected by glutathione-S-transferase genotype	Highlights the need for the presence of myrosinase (as in sprouts) for optimal SFN bioavailability
Hanlon et al, 2009 ⁴⁴	Determine the effect of repeated broccoli shake intake on SFN bioavailability	300ml of broccoli homogenate was ingested on 10 consecutive morning; dose was equivalent to 0.32 µmol/kg	Repeated ingested of broccoli homogenate did not alter pharmacokinetic profiles. $C_{max} \approx 12$ ng/ml plasma $T_{max} \sim 1.2$ hr	SFN is rapidly absorbed but repeated dosing does not alter plasma levels consistent with rapid excretion
Riedl et al, 2008 ⁴⁵	Investigation into the ability of orally administered SFN to increase ARE associated enzymes	Broccoli sprout homogenate (BSH) ranging from 25 – 200g equivalent to >6µmol glucosinolates/gram or 0.283 µmol/ml BSH with 25g fresh sprouts = 45g BSH. Therefore, 200g gives rise to 102µmol SFN.	Doses of 100-200g of BSH were capable of increasing phase II enzyme expression in nasal lavage cells. Data highlighted 60% bioavailability.	Dose response was present with 200g BSH significantly increasing phase II enzyme expression.
Myzak et al, 2007 ⁴⁶	Effect of Brocco sprout consumption of peripheral blood mononuclear cell (PBMC) histone deacetylase activity (HDAC)	Consumption of 68g of BroccoSprouts $\approx$ 105 mg SFN	HDAC activity was significantly inhibit at 3h posts consumption and appeared lowered at 6h. Levels returned to baseline at 24h.	Delivery of SFN via Broccosprout consumption sufficient to reduce HDAC activity in PBMCs.
Cornblatt et al, 2007 ¹¹	Examine the effect of ingestion of broccosprout homogenate on breast tissue SFN metabolites	Consumption of a 20ml filtered broccoli sprout drink containing 200µmol SFN, $\approx$ 50min prior to surgery	Breast removal occurred $\approx$ 93-103 min post dosage. SFN metabolites were readily found in removed tissue	Presence of SFN metabolites in breast tissue highlight bioavailability in this tissue following oral admin

Table 1.1: Pharmacodynamic and pharmacokinetic effects of SFN containing ingestants gained from pilot studies

1.3. Innate immune effectors and pathogen detection

As eluded to above, the key early effectors of the inflammatory response belong to the innate immune system and also those cells such as endothelial and epithelial cells which are exposed to the external environment and circulating pathogens respectively. Innate immune cells include cells of myeloid lineage, such as macrophages and neutrophils, which are well placed to form a first line defence system due to their omnipresence within body wide tissue parenchyma and within the vasculature circulation. Furthermore, their ability to move in and out of tissues allows them to constantly survey tissue and rapidly respond to any damage or foreign matter.

In order to rapidly detect tissue damage, cells of the innate immune system express a set of highly conserved pathogen recognition receptors (PRRs) named the toll like receptors (TLRs)⁴⁷. These allow rapid detection of non-self, through conserved molecular patterns called pathogen associated molecular patterns (PAMPs), and also host produced danger associated molecular patterns (DAMPs) such as high mobility group proteins and heat shock proteins (although some say the role of HSP's as DAMPs are contentious)^{48,49}. The homology and conservation of these receptors indicates the crucial role they play in the host defence system. To date, these TLRs comprise a family of at least 11 receptors, which detect both intra and extracellular prokaryotic and eukaryotic micro-organisms and are host specific⁵⁰. The fact that these receptors do not require *de novo* protein synthesis or clonal expansion allows swift activation of immune cell effector function on encountering these conserved PAMPs allowing the immune system to quickly initiate the perpetrators removal. Interaction with these receptors leads to activation of downstream signalling pathways ultimately activating gene transcription and alteration of inflammatory gene expression⁵⁰. The role of these groups of receptors, namely TLR4, will be relevant to this project due to their central role in detection of lipopolysaccharide and their expression on both leukocytes and platelets, two cell types whose biological activities will be investigated within.

1.3.1. Toll like receptor 4 (TLR4)

Due to this central role mentioned in the detection of lipopolysaccharide (LPS), the downstream effects of TLR4 activation is of importance to this work. The road to the discovery of TLR4 as the solitary transmembrane receptor for the detection of LPS spanned over 30 years at the end of the last millennia, where the work of several groups converged at this finding.

Several groups had been working on the *Toll* and it was known that the gene cassette, of which *Toll* was part of, played a key developmental role during *Drosophila* embryogenesis⁵¹⁻⁵³. Importantly, it

was becoming clear that there were corresponding homologs to these genes in mammals that played a key role in cell signalling downstream of cytokine activation. This gene cassette belonged to the Rel transcription factor family, which was known to be held in the cytoplasm, translocate to the nucleus to bind key DNA promoters and be activated by stimulation of a transmembrane receptor with homology to IL1R; furthermore, it was homologous to the Toll receptor^{51,53-55}. Subsequently, Lemaitre *et al*, found that these genes were induced in *Drosophila* following bacterial challenge⁵².

Using simple mammalian experiments, other groups had noticed the genetic variation associated with response to LPS injection in a few different mouse strains⁵⁶. It was known that the *Lps* gene was associated with alteration of the response to LPS challenge, however, it wasn't until work by Poltorak *et al.*, using a genetic approach to show that the C3H/HeJ and the C57BL/10ScCr mice strains had defective LPS responses due to allelic variation (causing missense and null expression) of a gene that linked to the *Tlr* gene that the transmembrane receptor was made apparent⁵⁷. In support of this, clinical data of a patient with a defective response to gram negative bacteria provided support for this data in a human setting⁵⁸.

Several key molecular players in the LPS detection pathway had been previously shown. The ability of LPS binding protein (LBP) to bind lipid A, and in effect, concentrate LPS within the plasma had been published⁵⁹. It had also been shown that CD14 played a role in LPS detection, as mice defective for this receptor had a greatly increased resistance to LPS challenge⁶⁰. Equally, it was known that this receptor lacked a cytoplasmic tail and was; therefore, likely to act as a co-receptor rather than via transmitting signals across the membrane to the cell cytoplasm⁶¹.

Together these formative studies combined to provide the cellular signalling pathways involved in LPS detection from the cell surface receptor, TLR4, to the gene upregulation that we now appreciate in response to gram negative bacteria (See Figure 1.2).

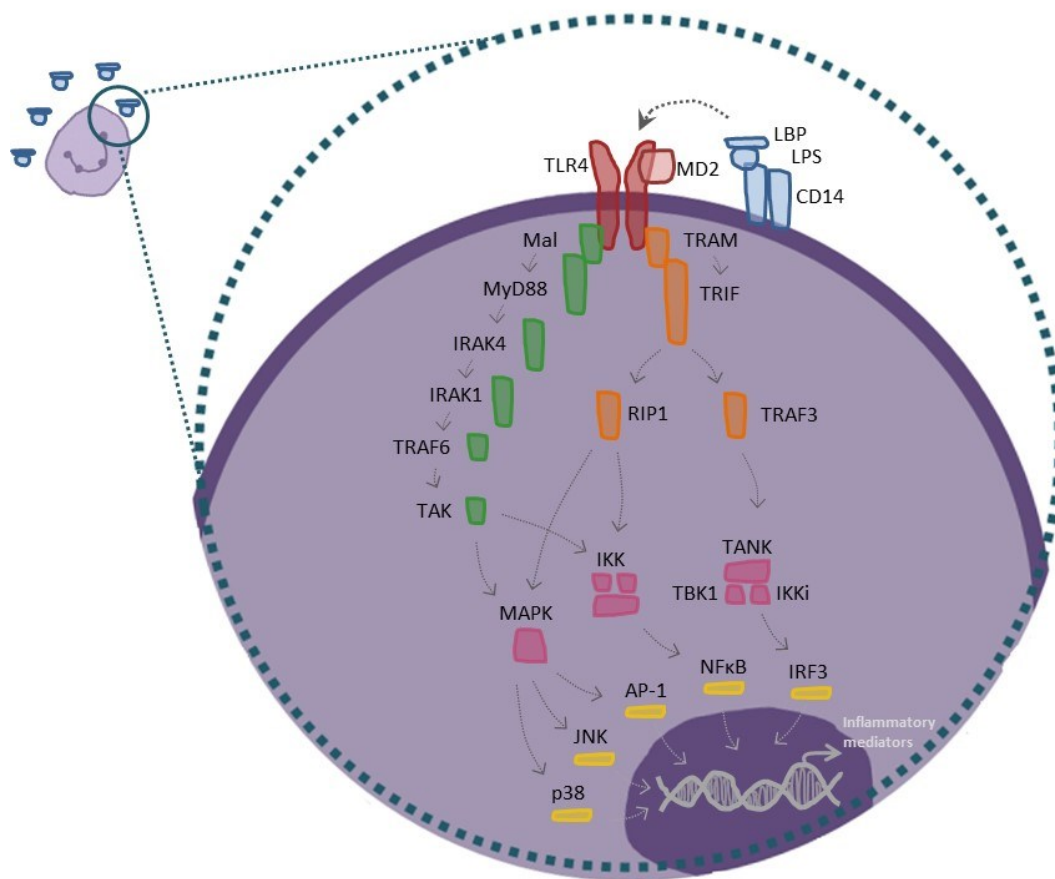


Figure 1.2: Intracellular LPS induced TLR4 signalling cascade

Diagrammatic representation of the intracellular signalling cascades stimulated downstream of TLR4 activation in the presence of LPS. Adapted from Lu et al, 2008⁶² (Gillespie, *unpublished*).

Since elucidation of this pathway, the use of LPS as a stimulant of the host innate immune response has provided the scientific community with a highly useful tool; both to ascertain the role of multiple signalling pathways during the inflammatory response and to investigate possible therapeutic agents. The role of TLR4 has also been highlighted as a key player during the inflammatory response outside of microbial recognition for example in pathologies involving ischaemia-reperfusion injury such as MI and stroke⁶³⁻⁶⁵. With reference to the brain, removal of TLR4 signalling by physiological knock out has been seen to ameliorate several of the inflammatory mediators, tissue damage markers and neurological outcomes associated with stroke pathology supporting the role for mal effects following its activation⁶⁶. In line with this, it was been seen that patients with higher TLR4 expression on peripheral blood cells following ischemic stroke showed a poorer prognosis with elevated

inflammatory markers (TNF α , IL-1 β , IL-6, ICAM1) and larger infarct development⁶⁷. Together, these findings highlight the role of TLR4 signalling in the activation of pro-inflammatory signalling cascades following exposure of the host to both endogenous and exogenous PAMPs and DAMPs.

1.4. Immune cells

1.4.1. Neutrophils

Neutrophils are cells of the myeloid lineage that are derived from the same precursor haematopoietic stem cell lineage that produces monocytes and macrophages. They are produced within the bone marrow, and are constantly released into the circulation to maintain a circulating level of 2.5-7.5x10³/μl (in man vs. 3-12x10⁴/μl in mice); however, their release is rapidly increased in response to infection⁶⁸. Like macrophages, neutrophils are phagocytic cells that circulate in the blood and can be recruited into tissues by various mediators (discussed below). They form the front line defence of the immune system and are the most numerous circulating cell of the immune system, accounting for approximately 50% of circulating leukocytes (in man vs. 10-40% of white blood cell population in mouse). Neutrophils have a lifespan of approximately 6hr once released from the bone marrow; yet, during inflammatory conditions their lifespan is significantly extended⁶⁹⁻⁷¹. Neutrophils are cleared, either within the tissue which they carried out their phagocytic role, or within the bone marrow, liver or spleen⁷².

Central to their phagocytic and antimicrobial function are the cytosolic granules and secretory vesicles that neutrophils harbour. Not only do these cytosolic reservoirs contain the neutrophils potent effector mediators, but they also serve as a pool of membrane receptors. Controlled release of these cytosolic stores serves to regulate neutrophilic effector function such as their recruitment to the inflamed tissue by allowing stimulant induced transmigration from the blood vessel into the tissue (see below). Timely release of these granules is paramount since their contents are highly destructive both to invading pathogens and to host tissues.

Primary or azurophilic granules are high in myeloperoxidase (MPO), a marker that is often used to suggest neutrophilic infiltration of tissues⁷³. MPO also plays an important role in the creation of cytotoxic oxidants via interactions with the products of NADPH oxidase. Alpha defensins and serine proteases are also important component of azurophilic granules and play an important role in the killing of internalised bacteria⁷⁴. These granules are mainly involved in intracellular killing following engulfment of bacteria, and are not readily released⁷⁵.

Secondary or specific granules contain important adhesion molecules and receptor, several of which are involved in leukocyte-platelet-endothelial cell interactions, such as CD11b/CD18 and urokinase plasminogen activator (uPA) and its receptor (R), uPAR⁷⁶. These two granule subsets have overlapping features in their peroxidase negative nature, and ability to release CD11b/CD18 (indicating its importance). Secondary granules also have a rich antimicrobial capacity, which differentiates them from tertiary (gelatinase) granule. Tertiary granules have a much greater tendency to be exocytosed, whereby they release several matrix degrading components aiding neutrophil recruit into and through the tissue^{77,78}.

The fourth, and smallest, granules are the secretory vesicles which have an endocytic phenotype and are released early following neutrophil activation⁷⁵. Detection of microbial products and pro-inflammatory cytokines induce their release⁷⁹. This leads to upregulation of cell surface receptors for initiation of the inflammatory cell recruitment and further detection of bacteria products, for example CD14 to aid LPS detection⁸⁰. In doing so, these granules adorn the neutrophil surface with several key proteins to increase microbial detection and enhance leukocyte-endothelial interactions without the exteriorisation of cytotoxic components harboured in other granules.

Finally, and most recently appreciated, is the ability of neutrophils to produce neutrophil extracellular traps (NETs). These NETs are externalised DNA fibres composed of externalised chromatin fragments harbouring cytosolic antimicrobial products⁸¹. They deliver the final wave of the neutrophil's arsenal to impede microbial invasion. NETs provide benefits in several ways; firstly, they form a physical barrier to capture invading pathogens and quell their dissemination; secondly, they localise bactericidal components released from the aforementioned mentioned granules thereby protecting surrounding tissue from their cytotoxic effects, and concentrating them to increase the attack on the pathogen^{81,82}. It is also worth mentioning that stimulation of the LPS:TLR4 pathway has also been shown to cause release of NETs, and that this can occur rapidly (within mins) and therefore, not act solely as a finale⁸². The crucial role that they play has been highlighted in simple, elegant experiments using DNase to remove their function, leading to a dramatic decrease in bacterial killing⁸¹. These NETs can also be released via interaction with activated platelets leading to a significant increase in the total area of NET production⁸². NET production does not however, play a purely beneficial role in the resolution of bacterial infection. Release of NETs within the vasculature has also been seen to provide a scaffold for platelet adhesion and activation, and thereby potentially promote vascular occlusion⁸². This highlights yet another facet of inflammatory cell activation with the potential for a dichotomous outcome.

Also of great importance to the physiological function of neutrophils is their ability to produce and respond to a plethora of cytokines and chemokines in their extracellular milieu. Production of hallmark pro-inflammatory cytokines including tumor necrosis factor α (TNF α), interleukin (IL-)1, IL-6 and interferon- γ (IFN γ) are stimulated over varying time points following neutrophil stimulation downstream of several cell surface receptors such as the TLRs. Their production is associated with various pro-inflammatory transcription factors such as NF κ B, p38 and ERK^{38,47}. These cytokines act to recruit further recruitment circulating immune cells, both additional neutrophils and other leukocytes such as monocytes. Various therapeutic agents that intercept these pathways either, at the initiating receptors, downstream kinases or transcription factors initiating their further expression, have shown therapeutic potential for decreasing the inflammatory response in an experimental setting. Many, however, appear to have little or no positive effect within the clinic potentially indicating the necessity of each cytokine in directing the developing immune response to inflammatory stimuli^{83,84}. Stimulation of neutrophils also leads to the production of mediators, for example IL-10, which have anti-inflammatory effects. IL-10's effects are transmitted via STAT3 and knock out of this pathway leads to pathogenic release of pro-inflammatory cytokine release and vastly increased mortality rates following exposure to LPS⁸⁵. Increasing knowledge of the role of cytokines within the immune response highlights the complex nature of both their temporal response and their interactions with one another in orchestrating both progression and resolution of inflammation.

1.4.2. Endothelial cells

The healthy endothelium exists as a confluent monolayer lining the inner surface of the vasculature, and thus, has an intimate association with circulating blood and its constituents. Under normal physiology and optimal flow conditions there is little interaction between the endothelium and cells circulating within the blood. The flow patterns within laminar sections of the vasculature mean that the majority of cell types are located within the central stream of the blood flow and therefore are not in contact with the vessel wall. To aid this, the healthy endothelium is a highly metabolically active interface. It is constantly producing a plethora of vasoactive compounds that act in a paracrine and autocrine fashion (e.g. nitric oxide (NO), prostacyclin and ADPase) on the endothelium itself, as well as both the cells within the vasculature and mural cells of the vessel wall⁸⁶⁻⁸⁸. Likewise, these cells are also able to release numerous mediators, such as NO and TNF α , that in turn alter endothelia function and therefore the physiology of the vasculature^{89,90}. These vasoactive products include chemical messengers such as nitric oxide (NO) and prostaglandins, as well as molecules involved in the clotting cascade such as plasminogen activators^{1,91}. Not only does production of these factors prevent leukocytes from interacting with the vasculature surface, it also maintains an anti-thrombotic

environment within the vessel⁹¹. Production of NO leads to anti-aggregatory effects on platelets which is supported further via release of heparin and tissue plasminogen activator which respectively have anticoagulant and fibrinolytic effects⁹². Together, the milieu of these bioactive factors serves to maintain a healthy interface between cells of the blood compartment and those of the vasculature.

Endothelial cells also contain several transcription factors that are known to be associated with athero-protection within the vasculature, such as Nrf2 and Klf2, which are increased in the presence of laminar flow leading to shear stress at the endothelial surface^{25,93}. Likewise, shear stress also alters the function of certain pro-inflammatory transcription factors, for example NFκB⁹⁴. Alteration of these transcription factors in line with flow is a relatively newly discovered phenomena but highlights how pathologies causing turbulent flow, such as atherosclerosis, can act to enhance inflammatory environments at vascular sites already at risk of inflammatory damage²⁵. Conversely, laminar flow and induction of these cytoprotective enzymes is associated with decreased inflammatory markers at the apical surface of the endothelium, and protects against endothelial dysfunction and the recruitment of inflammatory cells. Hence, these transcription factors not only protect the vasculature itself, but also the surrounding tissues. Recent evidence supports the ability of therapeutic intervention with agents such as SFN to mimic these effects and upregulate the induction of these anti-inflammatory transcription factors decreasing inflammatory cytokine/chemokine release, decreasing adhesion molecule expression and thereby altering leukocyte-platelet-endothelial interactions^{25,26}.

It is known that endothelial dysfunction is closely linked to the development of many cardio- and cerebro-vascular pathologies, as well as those initiated following bacterial infection⁹⁵⁻⁹⁷. Hence, therapeutic intervention that improves, or inhibits degradation of, endothelial function is an attractive target in reducing risk factors for the development of these diseases. Endothelial dysfunction not only leads to a decrease in the anti-inflammatory and anti-platelet factors mentioned above, but it also increases production of factors (e.g. TNFα, ICAM1) that actively promote leukocyte and platelet-endothelial interactions⁹². Loss of vasoactive mediators can also lead to alterations in flow so that cells are no longer held within the central stream of the blood flow, leading to a greater chance of interaction with the dysfunctional endothelium⁹⁶. This also leads to a dysregulation of the earlier mentioned transcription factors, whose expression are intrinsically linked with shear associated flow dynamics^{25,93}. Subsequently increased expression of adhesion molecules, such as ICAM1 and E-selectin thereby promotes recruitment of both platelets and leukocytes across the endothelium and into surrounding tissues^{25,93,98}.

1.4.3. Platelets

Platelets, once considered a rather inert sub-cellular fragment, are now thought of as a cell type in their own right with a wide array of biological functions. They are small, anucleated cells released from myeloid derived cells called megakaryocytes which are predominantly found in the bone marrow. Platelets are released through a process of budding off the megakaryocyte. Upon release, the mature platelet is approximately $2\mu\text{M}$ in diameter and discoidal in shape in their inactivate form. Their release, from megakaryocytes within the bone marrow into the circulation, makes them well placed to rapidly react in response to many physiological stimuli within the vasculature. They circulate within the vasculature for 8 – 10 days after which they are removed from the circulation by the spleen. Their normal concentration within the circulation is $2.5\text{--}7.5 \times 10^5/\mu\text{l}$ (in man vs. $3\text{--}10 \times 10^5/\mu\text{l}$ in mice).

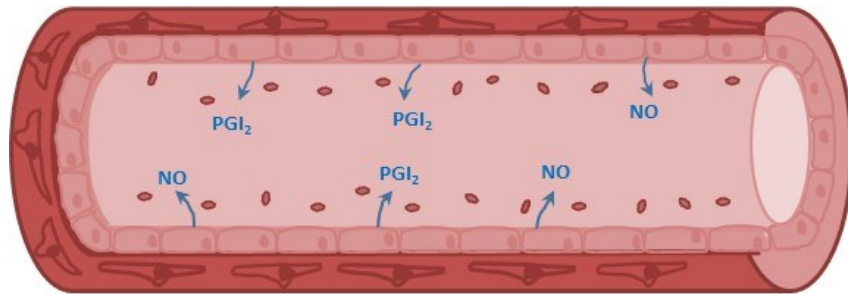
Platelets have a rather unusual form in that they are composed of an open canalicular system. This represents an extended network throughout the platelet cell body that significantly increases their surface area to volume ratio and therefore aids their role in reacting rapidly to thrombogenic factors within the vasculature; both via providing a greater surface to uptake circulating products and by increasing the area capable of releasing secreted products from the platelet⁹⁹. Platelets also have another characteristic ultrastructure known as the dense tubular system. This is thought to be derived from the megakaryocytic endoplasmic reticulum, and serves to regulate the platelets intracellular Ca^{2+} levels¹⁰⁰.

Another key structural feature of platelets are the alpha, dense and lysosomal granules. Alpha granules are the most abundant (10% platelet volume), and contain an array bioactive substances¹⁰¹. Some of the key bioactive substances they include can be grouped into; cell surface adhesion molecules such as P-selectin, vWF and other glycoproteins; growth factors including platelet derived growth factor, vascular endothelial growth factor and other factors involved in the coagulation cascade¹⁰². Dense granules are less numerous than there alpha counterparts (3-8 per platelet). They contain the largest intra-platelet ADP (50%) and calcium (60-70%) stores; as well as serotonin, pyrophosphates and non-metabolic ADP¹⁰³⁻¹⁰⁵. Finally, lysosomal granules are speculated to be remnants of the Golgi body collected from the megakaryocyte. These structures contain hydrolase enzymes which are released upon platelet activation and play a role in vascular repair¹⁰⁶. Release of the contents from all of these granules has effects not only on other platelets but also other cells in the vicinity such as leukocytes and endothelial cells, in order to begin repair the vascular damage and promote effect wound healing.

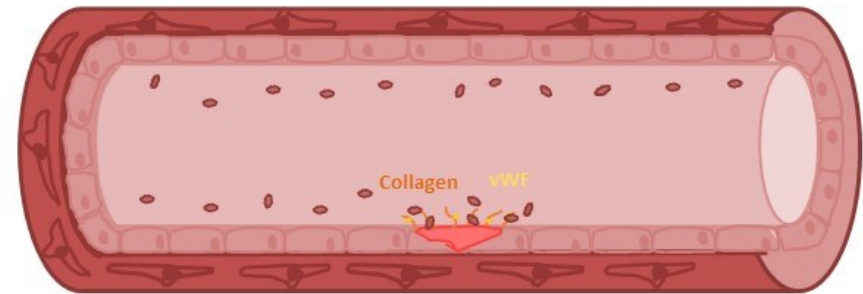
1.4.3.1. Haemostasis

The haemostatic response by platelets can be broken down in four key stages; platelet adherence, activation, aggregation and clot retraction. The aim of this series of events is to repair the damage to the vessel wall, whilst maintaining its patency (see Figure 1.3).

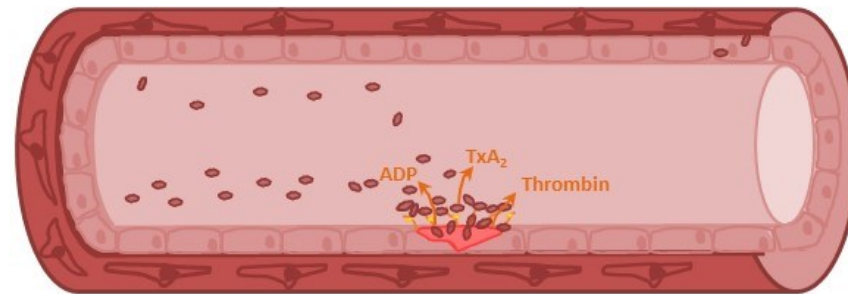
Under normal physiological conditions the primary initiator of platelet activation is exposure of collagen in the subendothelial matrix following damage to the wall of the vasculature. Adherence of platelets to collagen via GPVI and $\alpha_2\beta_1$ receptors induces outside-in signalling, changing the platelet from its resting discoidal phenotype to an activated 'amoeboid' like appearance^{107,108}. Alongside this induction of shape change, platelets also rapidly degranulate – releasing the constituents from the earlier mentioned granules, such as ADP, as well as newly formed mediators such as thromboxane¹⁰⁵. This allows an amplification of the response, recruiting and activating further platelets in the circulation. At this stage, several more cell surface receptors become activated. ADP released from α -granules interacts with the purinergic receptors P_2Y_1 and P_2Y_{12} to further activate and provoke shape change in subsequently recruited platelets^{109,110}. The P_2Y_1 receptor is thought to primarily activate shape change and the primary wave of platelet aggregation, since KO mice for this receptor showed increased bleeding time and a decreased sensitivity to ADP stimulated aggregation and thrombi formation¹⁰⁹. The P_2Y_{12} receptor on the other hand has been recognised to be responsible for secondary wave aggregation due to granule secretion, thromboxane production and also inside-out activation of the fibrinogen receptor¹¹⁰⁻¹¹². Furthermore, vWF, released from α -granules, is expressed at the platelet cell surface and bound to collagen, and due to its multimeric structure, serves as a further substrate for successive platelet recruitment via the GPIb/V/IX complex^{113,114}. In addition, expression of both P-selectin and PSGL-1 allows platelets to roll along the endothelium and further interact with other platelets, as well as leukocytes¹¹⁵. Thrombin, released as a product of the coagulation cascade, further activates recruited platelets via the PAR1 and PAR4 receptors, as well as breaking down fibrin to release fibrinogen¹¹⁶. This acts to stabilise the forming clot and cross links the aggregated platelets via $\alpha IIb\beta_3$ receptor¹¹⁷. This recruitment of platelets to the sites of vascular damage in some ways mirrors the leukocyte adhesion cascade which will be discussed later (Section 1.5). Not only does platelet recruitment use similar families of receptors, such as the immunoglobins (GPVI receptor) and integrins ($\alpha_2\beta_1$), but activation of outside in signalling, as known to occur in leukocytes, is also involved in further platelet activation. Early platelet rolling, allows this 'outside in' signalling phenomena to prime platelets, for example via increasing integrin affinity, leading to subsequent stable adhesion of the platelets a process that is also key to the leukocyte adhesion cascade.



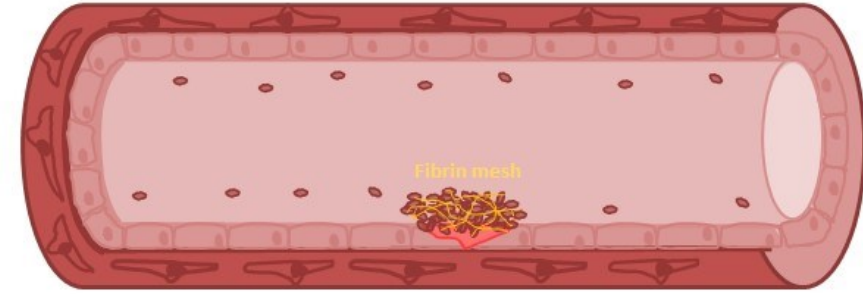
1. Healthy Blood vessel: Endothelium releases NO and PGI₂, maintains vessel patency + inhibits platelet activation



2. Damage Blood vessel: ↓ NO and PGI₂, P-selectin expression, ROS and cytokine production, vasoconstriction + exposure of collagen and vWF



3. Platelet activation: Platelets adhere via GPVI + $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$ and GPIb/IX/V, and release of platelet granules amplify recruitment



4. Clot stabilisation: Platelets become cross linked with fibrin leading to the formation of the haemostatic plug, resolving the damage

Figure 1.3: Platelet adhesion cascade

Overview of the platelet adhesion cascade following damage to the vascular endothelium. Adapted from Brass, Understanding and Evaluating Platelet Function¹¹⁸. (Gillespie, *unpublished*)

1.4.3.2. Thrombosis

Where haemostasis represents a vital and lifesaving physiological function of platelets, thrombosis represents the flip side of the coin whereby pathological activation of platelets is inappropriate and potentially life threatening. The inability of platelets to differentiate between vasculature damaged by injury, and exposure of pro-aggregatory sub endothelial molecules associated with pathologies such as atherosclerosis, leads to pathological platelet activation and issues with vascular occlusion and potential tissue infarction.

Thrombus formation has been shown to vary depending on the blood vessel in which it occurs. Thrombi found within higher pressure blood vessels of the arterial system, e.g. aorta, are referred to as 'white' and have a relatively higher leukocyte contents; whereas, thrombi formed within lower pressure vessels such as the venous system and the cardiac atria, are referred to as 'red' clots, due to their high erythrocyte and fibrin¹¹⁹. The disparity in properties of these clots is of clinical interest for optimal therapeutic intervention¹¹⁹.

1.4.3.3. Platelet cellular interactions

Activation of platelets is associated with phenotypic alteration: morphological change, alteration in cell surface receptor expression and release of chemotactic products. Expression of P-selectin is needed in order to allow platelets to adhere and form a stable clot following activation¹²⁰. This increased expression of cell surface adhesion molecules, such as P-selectin released from intracellular Weibel-Palade bodies, promotes interactions between platelets and activated leukocytes and endothelial cells^{121,122}. These interactions also allow platelets to act as a bridge to recruit circulating leukocytes to the sites of vascular damage¹²³. Indeed, leukocytes circulating in the vasculature associated with platelets are seen to have increased adhesion molecule expression, supporting the view that platelets enhance the likelihood of leukocyte-endothelial interactions¹²⁴. Additionally, platelets that are adhered to the vascular endothelium release several pro-inflammatory mediators including IL-1, CD40L and CCL-5 which further potentiate recruitment of circulating leukocytes via activation of the endothelium¹²⁵⁻¹²⁷. Furthermore, stable platelet aggregates adhered to fibrillar collagen have been shown to be capable of capturing passing leukocytes¹⁰⁷. Of further interest, it is becoming apparent that platelets do not just play a role in aiding leukocyte responses within the vasculature. Emigrated platelets have been found within inflamed tissues, raising the possibility that they further function to guide leukocytes within the inflamed tissues; possibly through production of further inflammatory cues or mediators¹²⁸. This could be one possible reason when platelet depletion has previously been shown to decrease leukocyte transmigration¹²⁹.

By inhibiting platelet-leukocyte interactions several groups have shown these interactions play a role in driving vascular inflammatory responses. Following peripheral immune stimulation, leukocytes have been seen to roll along cerebral pial venules 'paved' with platelets¹³⁰. Reducing platelet activation and decreasing platelet-leukocyte interactions via therapeutic aspirin, led to a decrease in leukocytes interacting with the cerebral microvasculature¹³⁰. This finding emphasises the effect platelets have on leukocyte-endothelial interactions, and highlights the inhibition of these interactions as an attractive therapeutic target. Similar results have also been found in other inflammatory conditions, both centrally and in the periphery, whereby platelet and leukocyte interactions are dependent on adhesion molecule expression and inhibiting these interactions dampens the ensuing inflammatory response^{124,131}. In support of this, appropriate widespread use of antiplatelet and anticoagulant medicines within the clinic makes apparent the marked benefit that inhibiting platelet activation can have for the therapeutic benefit to patients at risk of inflammatory vascular occlusion^{119,132}. This has the added benefit that reducing platelet activation lessens leukocyte activation and therefore further decreases inflammatory risks within patients^{133,134}.

When appropriate spatial and temporal control of this response are in place, platelet activation during haemostasis is a crucial event for the maintenance of blood vessel integrity. However; the benefits of these platelet targeted medicines highlight the devastating effect that inappropriately activated platelets can cause in several vasculopathies (e.g. atherosclerosis, MI and stroke)¹³⁴⁻¹³⁷. Investigating the ability of SFN to affect there physiological responses will therefore form an interesting adjunct to the *in vivo* leukocyte-endothelial interaction work conducted in this thesis.

1.5. Leaving the vasculature

As alluded to above, it is a crucial function of normal physiology and the developing inflammatory response that leukocytes are able to emigrate from the vascular compartment, cross the basement membrane and enter into inflamed tissues (see Figure 1.4). This is a key phenomenon underlying the onset of inflammation; visualisation of this phenomena using IVM will therefore be used as one of the main parameters to investigate the effects of SFN on leukocyte-endothelial interactions during the inflammatory response within the cerebral microvasculature. The importance of these intravascular cellular interactions are highlighted by patients suffering from leukocyte adhesion deficiency syndrome, a group of clinically related diseases whereby patients' neutrophils have an inability to transmigrate into tissues leading to problems such as leucocytosis, recurrent bacterial infections and delayed wound healing¹³⁸.

The ability of leukocytes to enter in to tissues has been appreciated for nearly 200 years, and work by early pathologists such as Duchrochet, Addison, Elrich and Metchnikov exemplify this¹³⁹. Cohnheim was one of the first pathologists known to actively observe leukocytes emigrating from the blood into the tissue of the frogs tongue using an early form of intra-vital microscopy¹³⁹. Since then, the cells, adhesion molecules and inter- and intra-cellular pathways involved in the leukocyte recruitment cascade has vastly evolved. A wealth of novel technologies has aided our understanding; from the use of genetically engineered mice to improvements in the microscopes and software we use. Together, these technologies have allowed us to expand upon the previous phases of leukocyte-endothelial interactions termed rolling, adherence and transmigration to a multistep cascade with a much greater complex series of events and stages involved. IVM has been a key technology in our developing understanding of leukocyte-endothelial interactions during inflammation, and will therefore be used as a key tool to investigate SFN's effects on these important cellular interactions.

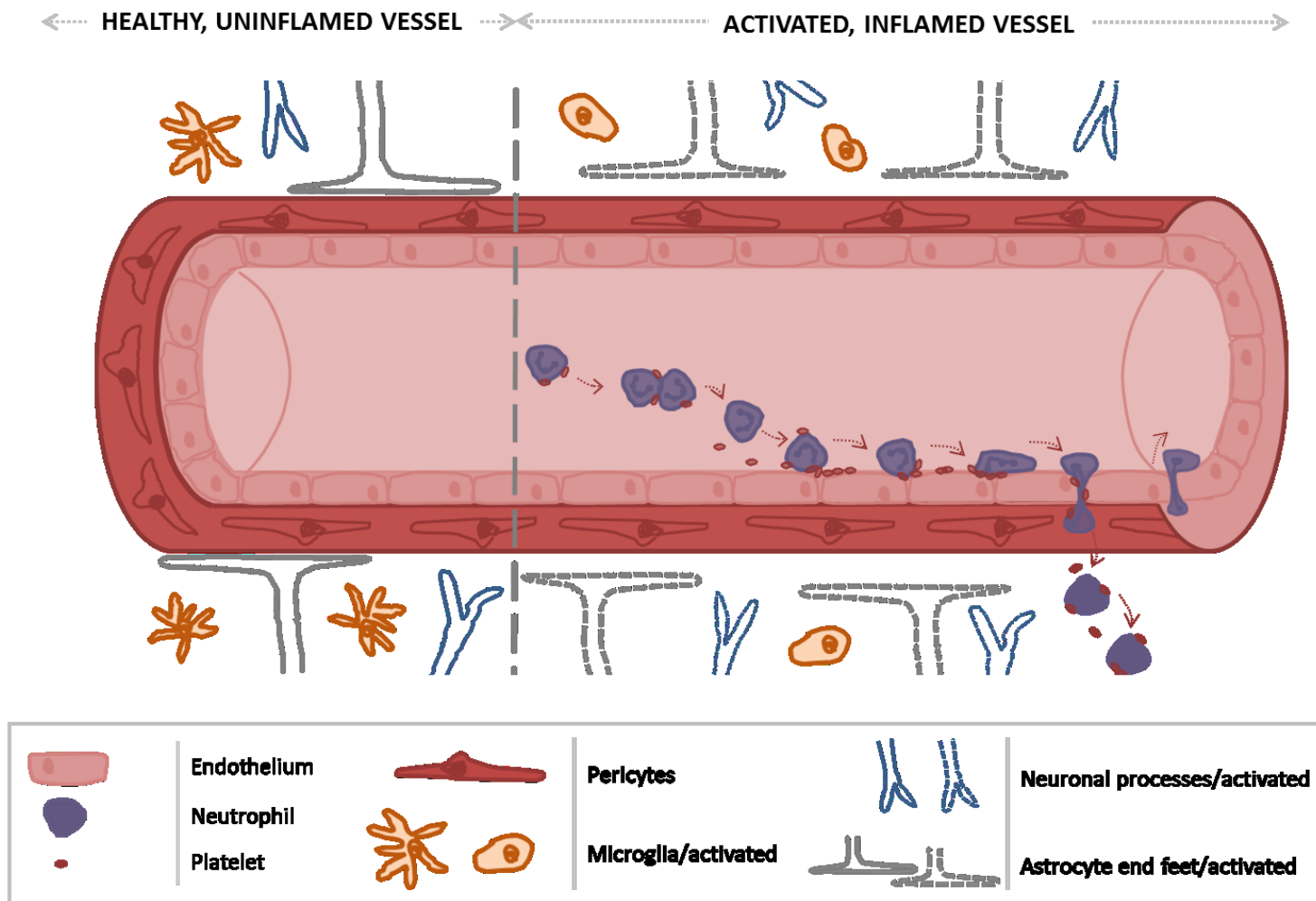


Figure 1.4: Leukocyte adhesion cascade.

Diagrammatic interpretation of a pial vessel showing the absence of cellular interactions in a healthy, uninflamed venule (left segment) versus the hallmark leukocyte adhesion cascade associated within an activated, inflamed venule (right). (Gillespie, *unpublished*).

Initial interactions require that both leukocytes and endothelial cells express a family of receptors known as selectins (See Table 1.2). Selectins are single chain glycoproteins with a transmembrane domain and intracellular cytoplasmic tail. Leukocytes (L-selectin) ubiquitously express selectins, however, under normal physiology endothelial selectins (E and P-selectin) are stored in intracellular α -granules of platelets and Weibel-Palade bodies within endothelial cells^{79,101}. Following an inflammatory stimuli, endothelial cells and tissue resident macrophages release classic pro-inflammatory cytokines such as TNF α , IL-1 β and IL-6. These released cytokines bind receptors on the endothelial cell surface causing the release of cytoplasmic granules leading to a sequential upregulation of cell surface selectins. All selectins bind the ligand P-selectin glycoprotein ligand 1 (PSGL-1) which is expressed by leukocytes, platelets and endothelial cells allowing cells to undergo both hetero and homotypic cellular interactions¹⁴⁰. It appears that these interactions are important, and that the binding of platelets to both endothelial cells and leukocytes can promote leukocyte recruitment into the inflamed tissue. For optimal selectin-PSGL-1 binding to occur, PSGL-1 must be appropriately glycosylated¹⁴¹. Activated leukocytes localise selectins to microvilli tips in order to improve initial presentation to endothelial cells and therefore promote cellular interactions^{142,143}.

Formation of these selectin based bonds are reversible and allow the initial tethering of leukocytes along the apical border of the inflamed endothelium. This rolling also allows exposure of the leukocyte to further chemokines, e.g. CXCL1, that are displayed on the surface of the endothelium on the glycocalyx. Chemokines are small proteins of approximately 8-10kDa, released by various cell types, that bind to cell surface receptors and can induce cellular movement along their concentration gradient¹⁴⁴. The glycocalyx serves to trap and present chemokines to interacting leukocytes, creating a higher local concentration at the inflamed site and thereby supporting leukocyte-endothelial interactions¹⁴⁵. This local environment acts to further increase both the affinity and avidity of selectin bonds and prime for the subsequent slow rolling and adhesion phase of the leukocyte recruitment cascade. Binding of selectins and chemokine receptors activates intracellular kinases such as Src and Syk triggering a network of downstream signalling mediators (PLC γ , PI3K, and p38 MAPK); this leads to cytoskeletal rearrangement and priming of the next family of leukocyte-endothelial mediators, the integrins¹⁴⁶⁻¹⁴⁸.

Integrin ligand specificity is dictated by the specific pairing of various members of the α and β subunits which pair to make a functional integrin molecule. Particular to neutrophils, and this next phase of leukocyte-endothelial interactions, are the β_2 integrins (namely LFA1 and Mac1 for neutrophils) and their ligands ICAM1, and ICAM2 of the immunoglobulin superfamily ((Ig) see Table 1.1). Under non-

inflamed conditions β_2 integrins are held in a low affinity state with their extracellular domain in a bent conformation that allows only weak interactions with rolling leukocytes¹⁴⁹. Furthermore, the expression of ICAM1 is low during normal physiological conditions, meaning there is little chance of Ig: β_2 interactions. Upregulation of ICAM1 expression has been shown to occur as early as 4hr in presence of inflammatory cytokines such as TNF α , supporting greater interaction of ICAM1 and β_2 integrins¹⁵⁰. This is further supported by the earlier mentioned activation of inside-out signalling via chemokine receptors¹⁵¹. A role of PLC, GTPases and cytoplasmic proteins that interact within actin-binding proteins have all been suggested to have a role in this inside-out signalling event¹. This signalling alters the conformation of the β_2 integrins in several ways. Firstly, reorganisation of intracellular cytoskeleton and/or ligand binding, allows a spatial separation of the α and β chains of the integrins¹⁵². This is necessary to allow extension of their conformation. Secondly, combining this with breaking of salt bridges between the intracellular tails 'unwinds' the extracellular domain, thus, exposing the β -propeller head of the α chain and straightening of the β_2 chain^{152,153}. The sum of these changes leads to exposure of an ICAM1 binding site with much higher binding affinity, mostly mediated by LFA1. Members of the cytoskeletal proteins that associate with actin, such as Talin and Kindlins, then rearrange to support this new extended confirmation, and link the integrin tails to the cells cytoskeleton¹⁵⁴. These changes markedly slow the speed at which the leukocyte rolls along the vascular endothelium, leading to slow rolling and arrest. While it has been suggested that LFA1 mediates the beginning of leukocyte adhesion within the inflamed vessel, it seems that Mac-1 is more predominant in the intravascular crawling that proceeds transmigration out of the vessel^{155,156}. The process of intraluminal crawling also allows the leukocyte to move along the inner surface of the vessel in order to find optimal sites of transmigration.

These cell adhesion molecule mediated leukocyte-endothelial interactions can be visualised using IVM and later analysed off line whereby cell numbers can be counted and the speed at which leukocytes are rolling down the endothelium can be calculated as a measure of the developing or ongoing inflammatory response. High levels of cellular adhesion are thought to highlight the presence of greater strength in leukocyte-endothelial interactions and therefore the presence of a more inflamed environment.

Once adhered, leukocytes may then emigrate via either a transcellular (through the endothelial cell) or paracellular route (between endothelial cells) out of the vessel. Although less well appreciated until recent years, it appears that the transcellular route of transmigration may account for up to one fifth of transmigrating cells in certain conditions¹⁵⁶. When moving between endothelial cells, leukocytes

use some adhesion molecules similar to those used at the apical surface of the endothelial cell, for example LFA1 and Mac1 (neutrophil) interact with JAMA-C (endothelial cell); as well as other homotypic interactions between CD99 and PECAM1¹⁵⁷. Clustering of ICAM1 leads to the formation of focal microdomains named endothelial adhesive platforms¹⁵⁸. These are supported and reinforced by further recruitment of actinins to ICAM1 cytosolic tails^{159,160}. Intracellular signalling associated with ICAM1 binding at endothelial cell junctions leads to phosphorylation of another junctional protein, VE-Cadherin, which destabilises endothelial homotypic interactions further permitting leukocyte passage between the cells^{161,162}. This phenomena can be visualised in certain tissues such as the mesentery and cremaster muscle, but is more difficult in tissues such as the brain due to the depth and nature of the tissue.

Once leukocytes have breached the endothelial cell monolayer, they must then cross the basement membrane (BM) and associated pericytes that are located within it. It has been seen that within the BM there are areas of less dense expression of BM matrix protein expression termed low expression regions (LERs). These LERs are associated with inter-pericytes spaces, suggestive of interplay between the pericytes and formation of the BM in the extravascular microenvironment¹⁶³. It is thought that these are used by leukocytes, neutrophils in particular, to aid their passage through the BM. This may explain why leukocytes have been visualised crawling along the abluminal surface of the vasculature; firstly, 'deciding' whether to enter the tissue before potentially re-entering the vessel, and secondly, 'searching' for permissive sites at which to cross the BM¹⁶⁴. Indeed, abluminal leukocytes have been visualised crawling marked distances along pericytes before crossing at LER sites⁹⁰. Interestingly, neutrophils are seen to use similar adhesion molecules to crawl along pericytes as those used on the luminal surface of the vascular wall. Both ICAM1 and Mac1 are upregulated following inflammatory stimuli, and blocking these hinders neutrophil-pericyte interactions^{89,90}. Neutrophils have also been shown to use degradative enzymes, released from intracellular granules, in order to penetrate through the BM. This is supported by the presence of laminin positive neutrophils in the post BM tissue, and also inhibition of BM penetration following treatment with a of neutrophil elastase inhibitor¹⁶⁵. Of interest, it has also been shown that leukocyte migration is not a uni-directional event. It is possible for leukocytes to exit the vessel, survey the abluminal environment and then cross back through the endothelium¹⁶⁴.

Once within the tissue, leukocytes migrate along chemokine gradients released by resident cells; IL-8 is one of the major chemotactic agents used by neutrophils¹⁶⁶. Alterations in the phenotype of neutrophils within the tissue reduce further recruitment of leukocytes. This includes activation of

suppressor of cytokine signalling (SOCS)3 to prevent on-going pro-inflammatory gene induction by factors such as signal transducer and activator of transcription (STAT)3. The importance of this has been highlighted by knockout of SOCS3 leading to generation of neutrophils with a hyperactive phenotypes¹⁶⁷. Also, more recently the role of microRNA induced in association with inflammatory stimuli such as LPS has highlighted their role in negative feedback of inflammatory mediators such as NFkB^{168,169}. This resolution of inflammation concomitant with tissue damage is vital to the temporal regulation of the inflammatory response.

<i>Family</i>	<i>Adhesion molecule</i>	<i>Other Name</i>	<i>Ligand</i>	<i>Cellular expression</i>	<i>Cellular location</i>	<i>Role</i>	<i>Consequence of KO/blockade</i>
<i>Selectin</i>	P-selectin	CD62P	PSGL-1	Platelets Endothelium	Luminal/Apical	Initial L-E, P-E and P-L interactions. Cell activation.	Reduced cell rolling ¹⁷⁰
	E-selectin	CD62E ELAM1 LECAM-2	PSGL-1	Endothelium	Luminal/Apical	Initial L-E and P-E interactions. Cell activation.	Additive effects when knocked with P-selectin, less marked effects on leukocyte rolling extravasation alone ¹⁷¹
	L-selectin	CD62L	PSGL-1	Leukocytes	Microvilli tips	Initial L-E, L-L and P-L interactions. Cell activation.	Decreased cell rolling and extravasation ^{172,173}
<i>Mucin</i>	PSGL-1	CD162	Selectins	Platelets Endothelium Leukocytes Monocytes	Cell surface	Initial L-E, P-E and P-L interactions. Cell activation.	Protection from thrombus formation ¹⁷⁴
	ESL-1		Selectins	Endothelium	Cell surface		?
<i>Ig Super Family</i>	ICAM-1	CD54	Integrins	Endothelium	Luminal/Apical	Reduced velocity rolling, firm adhesion and cell activation	Reduced leukocyte extravasation and resistance to LPS challenge ¹⁷⁵
	ICAM-2	CD102	Integrins	Endothelium	Junctional	Paracellular cell migration	Similar adherence to WT. Reduced transmigration in model and stimulus dependent manner ^{176,177}
	PECAM-1	CD31	PECAM-1	Endothelium Neutrophil	Junctional	Paracellular cell migration	Similar adherence to WT. Reduced transmigration in response to IL1 β . Leukocytes accumulate between EC's and BM. ¹⁷⁸ Again, model dependent ¹⁷⁶

Integrins	CD99	MIC2	CD99	Endothelium Neutrophil	Junctional	Paracellular cell migration	As above
	JAM-A	JAM-1 PAM-1 CD321	JAM-A LFA-1	Endothelium	Junctional	Paracellular cell migration	Reduced leukocyte transmigration in a stimulus dependent manner ¹⁷⁹
	JAM-B	JAM-2 VE-JAM CD322	LFA-1	Endothelium	Junctional	Paracellular cell migration	?
	JAM-C	JAM-3	MAC-1	Endothelium	Junctional	Transcellular/ Paracellular cell migration	Disrupts transendothelial migration, increases no. of hesitant leukocytes ¹⁶⁴
	LFA-1	$\alpha_L\beta_2$ CD11a/CD18 CR3(β)	Ig Family	Neutrophil Platelets	Luminal/Apical Abluminal	Reduced velocity rolling, firm adhesion and cell activation Cell migration	Reduced platelet and leukocyte adherence following I/R ¹⁸⁰ .
	MAC-1	$\alpha_M\beta_2$ CD11b/CD18 CR3(α)	Ig Family	Neutrophil Platelets	Luminal/Apical Abluminal	Reduced velocity rolling, firm adhesion, cell activation and paracellular cell migration	Reduced platelet and leukocyte adherence following I/R ¹⁸⁰ .

Table 1.2: Overview of adhesion molecules

Table reviewing current adhesion molecules known to participate in the leukocyte adhesion cascade (Gillespie, *unpublished*)

1.6. Immune effectors within the brain

Inflammation within the brain is central to several neurodegenerative diseases, and furthermore, it is now appreciated that activation of the peripheral immune system can also stimulate inflammatory responses within the brain. Although the brain is still classed by many as an 'immuno-privileged' site (due to the barrier function of the blood brain barrier (BBB)), our understanding of the effects of immune cell activation both at the BBB and within the brain parenchyma has greatly developed over the last half a century.

1.6.1. Microglia

Although still an area of contention, knowledge of the microglial as the resident immune cell of the brain has helped understand their role they play in both surveillance of the brain parenchyma and in response to the injured brain. Derived from myeloid progenitors early in the development of the brain, microglia are the only resident immune cell found solely in the CNS^{181,182}. It is worth noting that monocytic cells can be recruited from the periphery to the CNS and act almost identically to resident microglia, making it difficult to discriminate between the two.

Microglia display many receptors similar to those seen on peripheral immune cells such as MHC, TLRs and cytokine/chemokine/complement receptors¹⁸³. Under 'resting' conditions, they have a dynamic and highly motile phenotype, constantly surveying the brain parenchyma through the extension of their ramified cell bodies^{184,185}. This allows a vast area of the brain to be constantly monitored (estimates suggest the whole brain parenchyma could be covered within a few hr¹⁸⁶), and continual removal of cellular debris to be achieved. Following activation, microglia undergo rapid phenotypic changes, withdrawing their highly ramified processes to become amoeboid like cells with the ability to migrate through brain tissue to sites of damage. Once at the site of damage, they are able to act in a phagocytic manner, ingesting damaged tissue and releasing cytokines, chemokines and growth factors to recruit further microglia and peripheral immune cells^{183,186}. For this reason, activated microglia are highly indicative of an inflammatory reaction within the brain.

1.6.2. Astrocytes

Astrocytes are the most numerous glial cell within the brain, accounting for approximately 50% of the non-neuronal cell population¹⁸⁷. They have important functions in ionic, osmotic and neurotransmitter homeostasis with the brain parenchyma, as well as a supportive role to other cell types within the brain, namely neuronal cells. This is achieved in part by the astrocytes role in the 'neurovascular unit'; whereby, astrocytes aid not only the development but also maintenance of a functional BBB^{188,189}. Likewise, their ability to regulate glutamate levels, as well as their critical role in the metabolism of

glutathione and its precursors, are of paramount importance for the survival of neuronal cells. In the absence of astrocytes, neuronal cells are exquisitely more sensitive to the cytotoxic effects of glutamate due to the ability of astrocytes to remove glutamate from their microenvironment¹⁹⁰.

Additionally, astrocytes play a significant role in maintaining the redox environment within the brain. For an organ with such a high metabolic demand (20% of the body's O₂ consumption vs. 2% of its weight), it is important that the generation of reactive oxygen species (ROS) as a by-product of respiration is tightly controlled. The ability of neuronal cells to generate glutathione, a tripeptide essential for the protection against oxidative challenges within the brain, is markedly decreased in the absence of astrocytes¹⁹¹⁻¹⁹³. This is at least in part due their ability to release essential glutathione precursors to the neuronal cell population. They also play a similar protective antioxidant role through their participation in ascorbate cycling¹⁹⁴. On top of these functions, like microglia, they are capable of producing a plethora of both pro and anti-inflammatory, and vasoactive, molecules in response to various stimuli. The ability of astrocytes to alter local cerebral blood flow in response to neuronal activity has also been suggested, although, this response may be much more complex than previously appreciated^{195,196}.

1.6.3. Neurones

Neurones are the 'communicator' within the brain. They control nearly all bodily functions, and rely, in some way, on other brain cells to support their function. They predominantly reside within the grey matter of the brain and it is the pathological alteration of their function, and the associated devastating effects that these can have, which drives the bulk of neurotherapeutic research. Pathologies such as stroke and sepsis that lead to inflammation within the brain, either within that brain parenchyma itself or present peripherally leading to systemic effects, have been shown to significantly alter neuronal homeostasis and therefore function.

1.7. Vascular Cerebral inflammation

Cerebral inflammation has been shown to underlie many neuropathologies; Alzheimer's, Parkinson's, MS, Stroke and endotoxaemia to name but a few. What differentiates the inflammation seen in both Stroke and endotoxaemia from other cerebral inflammatory pathologies is that the response is initiated and largely driven by peripheral immune cells and mural cells of vasculature. For this reason, the aim of this work was that by establishing a reliable model of brain inflammation using either stroke or endotoxaemia as an initiating stimuli, the effects of SFN on the inflammatory intravascular interactions could be investigated. As highlighted above, agents and syndromes the effect these interactions alter the inflammatory outcome and may therefore be of therapeutic benefit in patients at risk of cerebral intravascular inflammatory events.

Both stroke and endotoxaemia are associated with marked leukocyte, platelet and endothelial activation. In the case of Stroke, platelet activation is central to the ischaemic event which drives the tissue infarction and subsequent inflammatory response associated with tissue reperfusion. Therefore, stroke risk factors involve pathologies associated with platelet activation such as atherosclerosis, atrial fibrillation and hypertension. Hence, targeting the inhibition of platelet activation by either anti-platelet agents such as aspirin and clopidogrel; or via anti-coagulants, such as warfarin, represent effective therapies in order to reduce the risk of stroke onset. Once a stroke has occurred, tissue plasminogen activator (tPa), represents the gold standard in current acute stroke treatment (a significant downfall with this therapeutic agent is the necessity for patients to receive treatment no longer than 6hr post tissue infarction¹⁹⁷). This, together with mechanical clot removal, allows recanalization of the blocked vessel and therefore reperfusion of the ischaemic tissue. Although these interventions are invaluable to restore blood flow in order to address the tissues oxygen deficit; it is also known that tissue reperfusion is associated with a significant inflammatory response which is concomitant with further tissue damage. This is not currently effectively targeted by therapeutic interventions. Therefore, an agent capable of effecting platelet responses, as well as immune cell activation represents a potential beneficial therapeutic avenue.

Clinically, patients suffering from endotoxaemia represent a different patient cohort from Stroke patients in that there is prevalence for patients to already be on a high dependency ward or intensive care unit. The trigger for endotoxaemia is infection with bacteria, both gram positive and gram negative, although gram negative bacterial infection is more commonly the cause. Infection leads to rapid activation of several cell types within the vasculature (via aforementioned TLR4 dependent pathway), including platelet, leukocyte and endothelial cells. Systemic inflammatory responses ensue

throughout the body; including the brain, which is affected by altered perfusion, cellular interactions at the blood brain barrier and release of inflammatory mediators.

The vascular inflammatory component and increased activation of several cell types, as well as TLR4 activation, is therefore common to both the pathology underlying stroke and endotoxaemia. The use of animal models and *in vitro* work offer an opportunity to study the effect of activation of these cells types, their subsequent interactions and also the ability of anti-inflammatory agent such as sulforaphane (SFN) to alter these responses.

Although these clinical pathologies arise from distinct patient cohorts, it is apparent that they share some common pathophysiological hallmarks such as TLR activation^{62,65}. On top of this, patients who have suffered from a bacterial infection are known to be at increased risk of subsequently having a stroke¹⁹⁸⁻²⁰⁰. Furthermore, some findings suggest that patients with a prior infection have also been suggested to present with greater neurological deficit on entering the clinic²⁰⁰. These findings suggest interplay between previous activation of the immune system and a possible priming effect increasing vulnerability for subsequent ischaemic episodes.

1.8. Models of cerebral inflammation

1.8.1. Stroke models

The ischaemic component of stroke is able to be modelled in animals via occlusion of arterial blood supply in order to enable investigators to study the whole body response to brain infarction. Models are categorised into global stroke; where all/the majority of the blood supply to the brain is occluded as would be the case following cessation of the heart beat or focal stroke; where a specific region or territory of the brain undergoes an ischaemic episode as would be the case following middle cerebral artery occlusion (MCAo).

Global stroke models are generally conducted in small rodents (mice, rats and guinea pigs), and involve clipping of the carotid (and in some cases vertebral arteries) leading to an interruption in cerebral blood flow (See Figure 1.5). These clips are left in place for 5min up to 1hr, and pathology during reperfusion is studied on removal of the clips. Focal stroke models involve a more technically difficult surgery leading to a localised, focal ischaemia. These are also most commonly conducted in rodents and may involve insertion of a filament into the middle cerebral artery (MCAO (see Figure 1.5)),

injection of a thrombogenic agent into the cerebral microcirculation or exposure and ligation of the MCA via a cortical window.

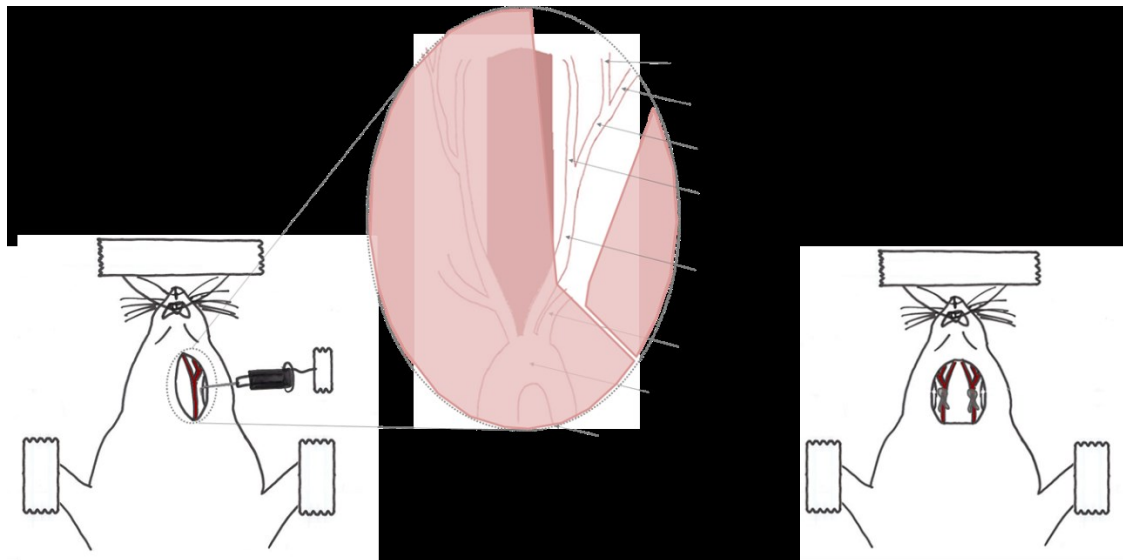


Figure 1.5: Surgical vessel isolation for global and focal stroke models

Diagrammatic representation of the isolated carotid arteries in mice. Right; represents clipped vessels in order to perform global ischaemia (BCCAO), and left; representing the isolation of the common carotid artery in order to introduce a filament into the left middle cerebral artery for a focal stroke model (MCAo). (Gillespie, *unpublished*)

MCAo is the most commonly used model, and can involve temporary or permanent filament placement. This model is thought to be the gold standard model due to its reproducibility, the ability to alter ischaemic times and reperfusion period to study the time course of damage onset and due to its ability to replicate some of the pathology seen in MCAo patients with in the clinic. Although this appears to be the model of choice for current stroke research considerable care must be taken in order to achieve reproducibility as variations in experimental protocol such as animal temperature and anaesthetic regimes can lead to significant alterations in research findings.

1.8.2. Endotoxaemia models

Models of endotoxaemia and the use of LPS have provided a valuable tool to study immune responses in both animal models and *in vitro* cell lines. Its addition to cultured cell lines has allowed carefully dissection of the cellular pathways activated downstream of TLR4 activation and the ability to study the effects of various potential therapeutic agents. Likewise, its use in animal models allows a whole body response to endotoxin to be studied enabling reactions throughout the vasculature and within

different vascular beds to be studied. Further, several groups have also reported data from human studies following the effects of 'microinjection' of LPS into healthy volunteers²⁰¹.

Specifically within the brain, inflammatory responses can be stimulated via direct injection of LPS into the brain parenchyma or following peripheral exposure to the bacterial product. Central injection leads to an inflammatory response primarily driven by activation of cells of the brain parenchyma, namely microglia. On the other hand, systemic exposure leads to a response driven by cells present within the vasculature and infected tissue. Effects of LPS injection in the periphery are dependent on treatment protocols such as dose and route of administration, but all lead to various alterations in haemodynamics, thermoregulation, immune cell activation, as well as altered physiology in many organs²⁰¹.

For the purposes of this project, a systemic challenge using *i.p.* injection was chosen in order to study the effect that activation of cells within the vascular component would have within the cerebral microcirculation.

The specific effects of SFN on leukocyte-endothelial cells interactions has not been previously visualised within the cerebral microvasculature. Furthermore, the effects that SFN has on platelets is only just beginning to be appreciated^{136,202,203}. How these alterations in platelet function may affect leukocyte-platelet interactions has also not been investigated. Owing to the aforementioned effects of platelet-leukocyte-endothelial interactions during inflammatory conditions, an agent that could affect the inflammatory profile of all of these cells may be highly beneficial in counteracting inappropriate inflammatory responses. Investigation into the ability of SFN to have these effects is therefore a major aim of the work herein.

1.9. Hypothesis

The hypothesis to be tested throughout the work presented in this thesis is that owing to the previously published ability of SFN to induce anti-inflammatory effects in several cell types;

Sulforaphane pre-treatment will induce protective, anti-inflammatory effects via Nrf2 leading to decreased intravascular cellular interactions and amelioration of the inflammatory profile associated with the inflammatory model established

1.10. Aims

In order to investigate this hypothesis the following aims will be addressed:

- 1. Establish a suitable model to induce and visualise cellular interactions within the brain following an inflammatory stimulus (Chapter 3)**

Leukocyte-endothelial interactions are known to be induced following inflammation and are a key process during the recruitment of leukocytes into inflamed tissues. Animals will undergo either MCAo (as a model of ischaemia induced inflammation) or receive an *i.p.* LPS injection (as a model of peripheral induced inflammation) to allow visualisation of leukocyte-endothelial interactions using IVM as a measure of the induction of a vascular cerebral inflammatory response.

- 2. Use the above model to investigate the ability of SFN to alter the leukocyte endothelial interactions during the vascular cerebral inflammatory response (Chapter 4)**

SFN has previously been found to have anti-inflammatory effects in several models. Following production and evaluation of a suitable cerebral inflammatory model, SFN pre-treatment will be used prior to inflammatory stimulation to examine whether this isothiocyanate is a suitable agent with which to alter leukocyte-endothelial interactions associated with cerebral inflammation. Peripheral cell counts will also be performed in order to observe whether total changes in cell numbers associated with the protocol could underlie any changes seen.

- 3. Use molecular *in vitro* approaches to study how SFN alters protein expression in brain tissue and cytokine production in response to inflammatory challenges (Chapter 4)**

Tissues taken from animals undergoing LPS and SFN treatment will be collected in order investigate the molecular changes underlying any changes associated with SFN pre-treatment, and cytokine

measurement will be used as a marker of the ability of SFN to alter the inflammatory profile associated with the model.

4. Investigate how SFN affects the functional response of specific isolated human immune cells in order to define potential cell specific effects (Chapter 4 + 5)

Stimulation of both leukocytes and platelets is known to contribute to the inflammatory response. Work with isolated cells types will aid understanding of the cell specific effects that are associated with any changes that may occur in the leukocyte-endothelial interactions seen in the animal model, and will also highlight how different cellular populations respond to SFN in isolation.

Chapter 2:

MATERIALS AND METHODS

2.1. Materials

Reagent	Supplier	Location
1,3,5, Triphenyl-tetrazolium formazan	Sigma Aldrich	Poole, Dorset, UK
Antisedan	Orion Healthcare	Berks, UK
Bupivacaine	Astra Zeneca	Luton, UK
Citrate dextrose solution	Sigma Aldrich	Poole, Dorset, UK
Corn oil	Sigma Aldrich	Poole, Dorset, UK
Fetal calf serum	Sigma Aldrich	Poole, Dorset, UK
Formalin solution	Sigma Aldrich	Poole, Dorset, UK
Fura 2-AM	Sigma Aldrich	Poole, Dorset, UK
Hanks Solution	Sigma Aldrich	Poole, Dorset, UK
Heparin, porcine sodium salt from intestinal mucosa	Sigma Aldrich	Poole, Dorset, UK
Histopaques (1077+1119)	Sigma Aldrich	Poole, Dorset, UK
Ketamine	Fort Dodge Animal Health	Southampton, UK
L-Glutamine–Penicillin–Streptomycin solution	Sigma Aldrich	Poole, Dorset, UK
LDH Cytotoxicity Kit	Roche Diagnostics	Germany
Lipopolysaccharide, 0111:B4	Sigma Aldrich	Poole, Dorset, UK
Pentobarbital Sodium	Merial Animal Health	Harlow, Essex, UK
Prostaglandin E₁	Sigma Aldrich	Poole, Dorset, UK
Rimadyl	Pfizer	Sandwich, Kent, UK
Roswell Park Memorial Institute (RPMI)	Sigma Aldrich	Poole, Dorset, UK
Saline	Baxters Healthcare	Thetford, Norfolk, UK
SFN	Sigma Aldrich	Poole, Dorset, UK
Turks Solution	Merck, Millpore	Darmstadt, Germany
Trypan Blue	Sigma Aldrich	Poole, Dorset, UK
Xylazine	Bayer	Newbury, Berkshire, UK

Table 2.1 Reagents used during experimental protocols

2.2 In vivo work

2.2.1. Animal work

All mice used in this project were 21-30g, male, C57Bl6 and purchased from Charles River, UK (except for mice used in photo-thrombosis model; Jackson Laboratory, USA). They were kept under ambient conditions; 19-23°C, 12hr light/dark cycle, free access to *ad libitum* standard chow and water with environmental enrichment provide in cages of up to 5. All experiments were conducted with appropriate licensing as per the Animals (Scientific Procedures) Act 1986/ EU directive 201/63 guidelines, and the revised Directive 2010/63/EU.

2.2.2. General surgery used in all intravital microscopy (IVM) studies

Mice were anaesthetised with Ketamine (150mg/kg) and Xylazine (7.5mg/kg), and placed on a heat mat with temperature maintained at 37°C ± 1°C. The skin overlying the right jugular vein was removed and the area surrounding the vein was blunt dissected clear. Surgical thread was looped under the vein and superior end was ligated to occlude the blood flow. A 27G needle tip was used to puncture the vein and a cannula was introduced for the later injection of Rhodamine 6G injection.

2.2.3 IVM protocol

Mice were then placed on a stereotactic board in a sphinx position with the head secured in a flat plane using ear bars. Skin covering the left parietal bone was removed, as was the underlying periosteum. Using a dental drill, a 5mm diameter cranial window was drilled and the bone was removed to expose the brain below. This was then bathed in saline and a coverslip was placed over the window. 100µl Rhodamine (0.02%) was injected through the cannula 5 min prior to visualisation using the intravital microscope with the CY3 filter in order to label circulating leukocytes.

2.2.3. LPS studies

Time response studies: Mice were treated with 0.5, 1 or 4mg/kg LPS and the cerebral microvasculature was visualised at 1, 2 or 4hr post LPS injection.

Dose response: Mice were injected with LPS at doses from 0.1, 0.25, 0.5, 1, to 4 mg/kg followed by visualisation of the cerebral microvasculature at 4hr.

At the end of all experiments blood was taken into heparinised syringes with a 25G needle and brains were collected following cervical dislocation for supporting *in vitro* work, snap frozen and stored at -80°C.

2.2.4.1. Middle cerebral artery occlusion (MCAo) model

Mice were anaesthetised with Ketamine (150mg/kg) and Xylazine (7.5mg/kg), and placed on a heat mat. Local application of bupivacaine was injected subcutaneously, and an incision was made in the skin overlying the left common carotid artery. The common carotid, external and internal carotid arteries, as well as the occipital and pterygopalatine arteries were bluntly dissected clear. Surgical thread was looped underneath the common artery and the inferior end of the common carotid artery was tied off. The superior end was clamped using a microvascular clip, and a small incision was made using micro-scissors into which the doccol was inserted. This was then guided up the internal carotid artery approximately 9-11mm in order to block the entrance to the middle cerebral artery. This was left in place for the entire ischaemic period, following which it was removed and silk around the superior end of the common carotid artery was occluded. The exposed area was then hydrated with saline, and the skin was sutured using a discontinuous stitch. The mice were injected with 500µl of saline for fluid rehydration and left to recover in a warm environment.

2.2.4.2 TTC staining

Following the specified reperfusion time period, animals were anaesthetised and blood was removed via cardiac puncture. The mice were then decapitated and the brain was removed, and placed in ice cold PBS for 15min. Using a razor blade and brain slicer, the brain was cut into 2mm coronal sections and then immersed in 2% TTC at 37°C for 15 min, followed by fixation in 10% formalin. Sections were laid out and photographed in order to quantify the infarct volume.

2.2.5. Photothrombosis model

Prior to experiments the intravital microscope was configured daily in order to ensure that the FITC light channel would expose a 100µm² area to $1.087 \times 10^{-3} \text{ W/cm}^2 (\pm 2\%)$ using a 175W Xenon light source. This intensity was set to following the photo-haemolysis of murine erythrocytes in a period of 5-6mins conducted to ensure consistency between FITC dextran lots for ongoing work.

Mice were then prepared as detailed above for the IVM experiments. Once the jugular had been cannulated and the mice had been placed on a stereotactic frame with the parietal cerebral cortex exposed, animals were injected with FITC-dextran (5% at 10ml/kg) via the cannula. This was allowed to circulate for 10min at which point mice were placed on the IVM. Appropriate vessels were selected (straight, non-branching, 30-45µm width for both arterioles and venules) on the bright field filter where possible. The filter was changed to the FITC channel. The timer was started and the thrombus formation was monitored. The point at which the platelets occluded the vessel lumen for a period of 30sec was recorded as the vessel occlusion time.

2.3 In vitro work

2.3.1. Cell fractionation

Brains were collected immediately following cervical dislocation, and snap frozen. Cortical regions were removed, and placed into a pre-chilled eppendorf. All samples were fractionated using the Actif Motif Nuclear extract kit (40010) as follows: Tissues were homogenised and incubated with 3ml/g tissue in hypotonic buffer supplemented (as per kit) with detergent (1%) and DTT(1%) on ice for 15min. Eppendorfs were transferred to a pre-chilled (4°C) centrifuge and spun at 850g for 10min. Supernatants were removed, and kept on ice for later pooling with a subsequent step. The pellet was then re-suspended in hypotonic buffer and incubated for 15min. 25µl detergent was added and samples were vortexed for 10 sec. Samples were spun down at 13000g for 10sec and pooled with the earlier fraction. These samples constituted the cytoplasmic fraction. For the nuclear fraction, the above pellet was resuspended in 50µl complete lysis buffer (as per kit), vortexed for 10sec and incubated on ice for 30min. Samples were re-vortexed and spun at 14000g for 10min. Supernatants were collected and saved as the nuclear cell fraction.

2.3.2. Protein quantification

All protein concentrations were quantified using Applichem Bradford Solution. 1µl of sample was added to 200µl of Bradford reagent. Samples were compared to a standard curve made using BSA (0-10mg/ml) in appropriate buffer. Colourimetric wavelengths were read at 595nm and compared against the known BSA protein concentration standard curve. All samples and curves were run in duplicate and the average was taken.

2.3.3. Western blots

All protein samples were quantified as above and used to ensure equal loading. Precast 10-20% Tris Glycine NU-PAGE gels were used with an XCell SureLock® Mini cell power pack. 10µl of SeeBlue® Plus2 prestained standard was used as a molecular ladder, followed by the quantified samples. Blots were run in Invitrogen Tris Glycine buffer for 2hr at 125V constant voltage. Gels were then removed from the cassette and transferred onto nitrocellulose membranes using an Invitrogen blotting module, with Invitrogen Running buffer (20% methanol) for 1hr at 25V. Blots were then blocked in 5% Milk TBSt for 1hr at room temperature, washed with TBSt for 10min on the rocker followed by incubation with the primary antibody in skimmed milk overnight at 4°C on the rocker. Membranes were washed with TBSt for 10min and incubated with horse radish peroxidase conjugated secondary antibodies for 1hr at room temperature. Pierce ECL western blotting substrates were then incubated with the membrane for 2min. In a dark room, X-ray films were then exposed to the membrane and films were developed.

2.3.4. Bead array

Blood samples from mice treated in the LPS studies were taken at the end of intravital microscope protocols using cardiac puncture into heparinised syringes with a 25G needle. Blood samples were spun down for 5min at 10,000RPM. Plasma was removed and snap frozen, then stored at -80°C. Plasma samples were then prepared as per BD Cytometric Bead Array kit protocol. The fluorescent intensities for each analyte (IL12p70, IL-6, TNF α , IL-10, IFN γ , MCP-1) in individual samples were then measured using a BDFACs Calibur Flow Cytometer. Cytokine expression was then quantified against a set of standards (0-5000pg/ml).

2.3.5. Nrf2 ELISA

Nuclear samples taken from the above cell fractionation studies were used in order to quantify nuclear Nrf2 expression across treatment groups. Samples were prepared as per Activ Motif Trans AM Nrf2 kit manual, and 8.5 μ g of nuclear protein were added per well in triplicate. Relative Nrf2 expression was then quantified from the colorimetric readout taken at 450nm.

2.4 Ex vivo work

2.4.1. Human neutrophil isolation

All reagents (Roswell Park Memorial Institute medium (RPMI), Fetal calf serum (FCS), Penicillin – L-Glutamine – Streptomycin, Histopaque 1077 and 1119) were placed in a sterile hood and bought to room temperature. 3ml of histopaque 1077 was layered over histopaque 1119. Blood was collected from NSAID free, healthy volunteers into a 60ml pull back syringe with 10% Sodium Citrate. Blood is then diluted 1:1 in RPMI containing penicillin (100units), L-glutamine (2mM) and streptomycin (0.1mg/ml) and 6ml is layered gently onto the histopaque gradient. This was spun at 400g for 30min at room temperature. The polymorphonuclear cell layer is removed, resuspended in 12ml RPMI and spun down at 400g for 15min. Cell pellets were then briefly resuspended in ice cold ddH₂O followed by addition of Hanks solution. Cells were spun down at 400g for 10min and then resuspended in RPMI (with penicillin (100units), L-glutamine (2mM), streptomycin (0.1mg/ml) and FCS (10%)). Cells are then washed in 12ml RPMI followed by spinning for 10min at 400g. Cell pellets were then resuspended, pooled and the cells are counted on a light microscope with Turks staining and a Neubauer Bright line haemocytometer. Cell numbers were calculated as follows:

$$\text{Cells number counted} \times \text{dilution factor} \times 10^4 = \text{Cells/ml}$$

Cells were then resuspended at 1×10^6 cells/ml in RPMI.

2.4.2. Human neutrophil stimulation

Cells were isolated and resuspended in RPMI with penicillin (100units), L-glutamine (2mM) and streptomycin (0.1mg/ml) as documented above. Cell suspensions were then aliquoted out onto two 24well plates at 5×10^5 cells/ml. Cells were then exposed to increasing doses of SFN (0.1-25 μ M) for 4hr followed by addition of LPS (0.1 μ g/ml) for 14h. Control wells contained untreated neutrophils. Plates were incubated at 37°C, 5% CO₂. At the end of incubations, plates were spun at 400g for 10min supernatants were collected for IL-1 β release analysis. Samples were snap frozen and stored at -20°C.

2.4.3. Neutrophil IL-1 β ELISA

At the end of neutrophil stimulation experiments aliquots of culture medium were taken and snap frozen and stored at -20°C. Samples were then thawed on the day of running the ELISA. These samples and serial dilutions of the provided known standard were then run in duplicate as per the manufacturers' instructions. The optical density of the unknown samples was plotted against a curve of best fit plotted from the known standards.

2.4.4. Neutrophil lactate dehydrogenase (LDH) Assay

Using the Roche cytotoxicity detection kit ^{PLUS} the following was conducted. Isolated neutrophils were suspended in RPMI with penicillin (100units), L-glutamine (2mM), streptomycin (0.1mg/ml) and 5% BSA, were pipetted across a 96 well plate, either with or without pre-incubation with SFN followed by LPS treatment. All samples were run in triplicate. Lysis buffer was added to the neutrophils marked for full lysis and untreated platelets were left for baseline LDH read outs. The plate was left on a shaker table at room temperature for 15min. Reaction mixture was added to each well, the plate was covered in foil and then left to incubate for a further 30 min. Stop solution was added to each well and the plate was then read at 490nm by a plate reader. Neutrophils treated with lysis buffer were taken as 100% LDH release and untreated neutrophils were used for baseline LDH release.

2.5 Platelet work

All blood was collected immediately prior to experimentation using venepuncture within the cubital fossa. All volunteers were healthy, aged 18 – 65 and NSAID free 2 weeks prior to blood collection. Blood was collected into 50ml pull back syringe using a 23G needle. The anti-coagulant used was acidified sodium dextrose (ACD, 1:9).

2.5.1. Platelet Isolation and Aggregation

Blood was aliquoted into 15ml falcon tubes, and spun down at 100g for 20min at room temperature. Plasma rich plasma (PRP) was then pooled in a 50ml falcon tube, and 5 μ g/ml PGE₁ and 12 μ l/ml ACD was added. PRP was then spun at 1400g for 10min to pellet the platelets. The supernatant was

discarded and the platelets were re-suspended in tyrodes-HEPES buffer with 10mM glucose (THB+), with 5µg/ml PGE₁ and 3ml ACD. Platelets were then centrifuged at 1400g for 10min. Platelets re-suspended in THB+ at 250 x 10³/µl. Platelets were then allowed to rest for 30min at 37°C. An appropriate volume of washed platelets were aliquoted into silicone coated cuvettes with stir bars, and placed in the light transmission aggregometer at 37°C under constant stirring conditions. Platelet reactivity was tested with 0.1U/ml thrombin to ensure reactivity before experiments were commenced. Aggregations were run for 3min following addition of various agonists.

2.5.2. Ca²⁺ signalling

Blood was spun down to the PRP stage, as above, then incubated at 37°C with CaCl₂ (200µM) and the ionophore Fura2-AM (5µM) for 30min in the dark. PRP was then supplemented with 5µg/ml PGE₁ and 12µl/ml ACD, and spun down at 1400 RPM for 10min. The platelets were then resuspended at suspended in THB+ at 250 x 10³/µl. Platelet reactivity was tested with 0.1U/ml thrombin to ensure reactivity before experiments were commenced.

2.5.3. Platelet LDH Assay

Blood was spun down to the PRP stage, and went through the first fast spin (1400g, 10min) then resuspended at in THB+ at 50 x 10³/µl. Using the Roche cytotoxicity detection kit ^{PLUS} the following was conducted. 100µL of washed platelets were then aliquoted out, either with or without pre-incubated of SFN, onto a 96 well plate in triplicate. 5µL of lysis buffer was added to the platelets marked for full lysis, and plate was left on a shaker table at room temperature for 15min. 100µL of reaction mixture was added to each well, the plate was covered in foil and then left to incubate for a further 30min. 50µL of stop solution was added to each well and the plate was then read at 490nm using a plate reader.

2.6 Data Analysis

2.6.1. Intravital microscopy videos

Videos recorded from mice undergoing intravital microscopy procedures were all recorded in real time but saved for off line analysis. Three parameters were quantified in order to evaluate the inflammatory response within these vessels (Figure 2.1). These included; the number of rolling cells, the cell rolling velocity and the number of adherent cells. The number of rolling cells was measured by counting the number of cells to pass a fixed point within the vessel (1.). The cell rolling velocity was measured via following a cells movement along the vessel wall and dividing the length it travelled by the time it took (2.). Finally, the number of adherent cells was counted as any cell that remained stationary within the

vessel for 30sec or longer (3.). All vessels chosen for analysis were straight and non-branching where possible.

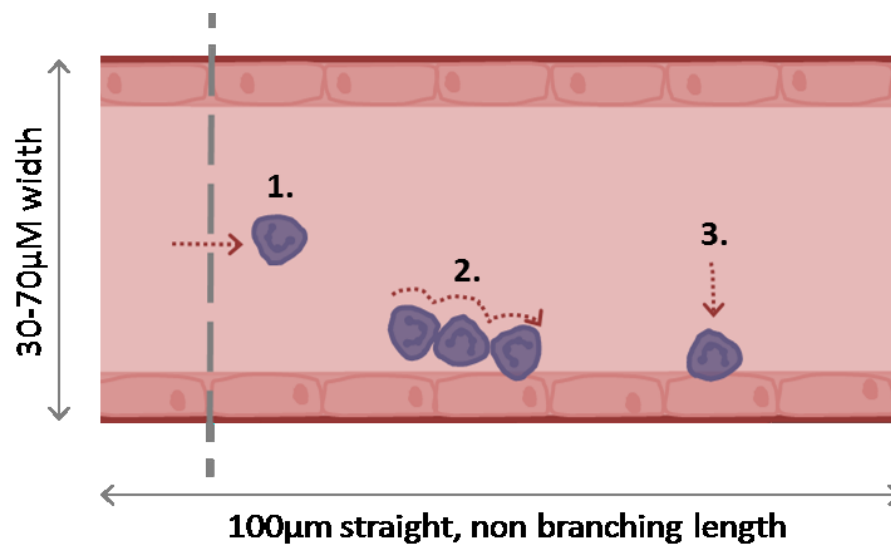


Figure 2.1: Diagrammatic representation of the parameters measured in order to quantify inflammatory responses within cerebral pial venules. (1. Number of rolling cell (mm^3/min) 2. Cell rolling velocity ($\mu\text{m}/\text{sec}$) 3. Number of adherent cells ($100\mu\text{m}$))

2.6.2. Statistical Analysis

All experiments were analysed using Prism Graph Pad statistical software. Experiments in Chapter 3 and 4 were analysed with non-parametric ANOVA with Dunnetts post hoc tests on mean $\pm$ SEM and experiments in Chapter 5 used parametric ANOVA $\pm$ SD. Power calculations for Chapter 4's *in vivo* experiments can be found in Appendix I.

Chapter 3:

METHODOLOGICAL DEVELOPMENT OF AN APPROPRIATE CEREBRAL INFLAMMATORY MODEL

3.1. Results

Outline

Much of the early work investigating the effects of SFN on cellular physiology had done so with respect to oncology with the use of cancer models and cell lines; more recent work suggests that some of the beneficial findings may be attributable to the ability of SFN to cause anti-inflammatory effects²⁰⁴. Subsequent studies have found that SFN's ability to induce Nrf2 within the vasculature promotes anti-inflammatory effects and, this in turn, provides protection against the development of atherosclerosis²⁵. Further work has shown that SFN crosses the BBB, a major hurdle for many therapeutics aimed at treating pathologies with the brain, and therefore, represented the possibility that SFN may be an attractive agent with which to target inflammatory pathologies within the brain²⁴.

The primary aim of this chapter of work was to set up an appropriate cerebral inflammatory model in order to investigate leukocyte-endothelial interactions within the brain:

1. Establish a suitable model to induce and visualise cellular interactions within the brain following an inflammatory stimulus

As highlighted in the introduction, stroke is a disease primarily driven by ischaemia caused by a blockage of the cerebral vasculature by a clot or embolus (approximately 85% of stroke patients suffer ischaemic stroke). The current dogma is that reperfusion of this ischaemic territory is vital in order to preserve the maximum amount of brain tissue, however, it is also associated with a significant inflammatory event – ischaemia-reperfusion injury (IRI). IRI is not currently effectively treated within the clinic. Through the use of the MCAo model of ischaemic stroke it was hoped we would recapitulate the pathology seen following ischaemic stroke in humans, enabling us to investigate the leukocyte-endothelial interactions underlying IRI. Using this model in combination with IVM would allow visualisation and analysis of these leukocyte-endothelial interactions. IVM provides a sensitive, real time technology with which to visualise and quantify the cellular interactions that underlie inflammatory responses as depicted in Figure 1.3. Collecting information on further parameters such as infarct development, cytokine production and neurological scoring would be used to gain more information on the pathophysiology associated with the model, as well as providing markers to relate the model to outcomes seen in humans.

MCAo protocol optimisation

The first protocol used involved insertion of a 210 μ M filament into the common carotid artery for a 60min occlusion period, followed by a 24hr reperfusion time point as had been previously published (although longer time points and permanent ischaemia have also been used)^{205,206}. This protocol was associated with unacceptable levels of mortality and therefore, with the aim of decreasing the mortality rate, the ischaemic period was decreased to a 30min ischaemic period. The effect of altering the body temperature was also tested alongside this new ischaemic period.

Reducing the temperature during ischaemia did appear to alter the leukocyte-endothelial interactions that occurred (Figure 3.1). Sham treated animals (identical surgical interventions without insertion of the filament) had few cells rolling through the pial vessels, and a low number of cells becoming adherent. In comparison, all animals that had undergone MCAo had increased numbers of cells rolling through the microvasculature and were doing so at a relatively slowing rolling velocity (25-40 μ m/sec; Figure 3.1b). The number of adherent cells within the pial vessels was altered by the temperature during ischaemia. Animals that were maintained at 35°C had low levels of adherent cells that were comparable to those seen in sham operated mice (Figure 3.1c). However, mice that had their temperatures maintained at either 36 or 37°C had a greater number of adherent cells (Figure 3.1c).

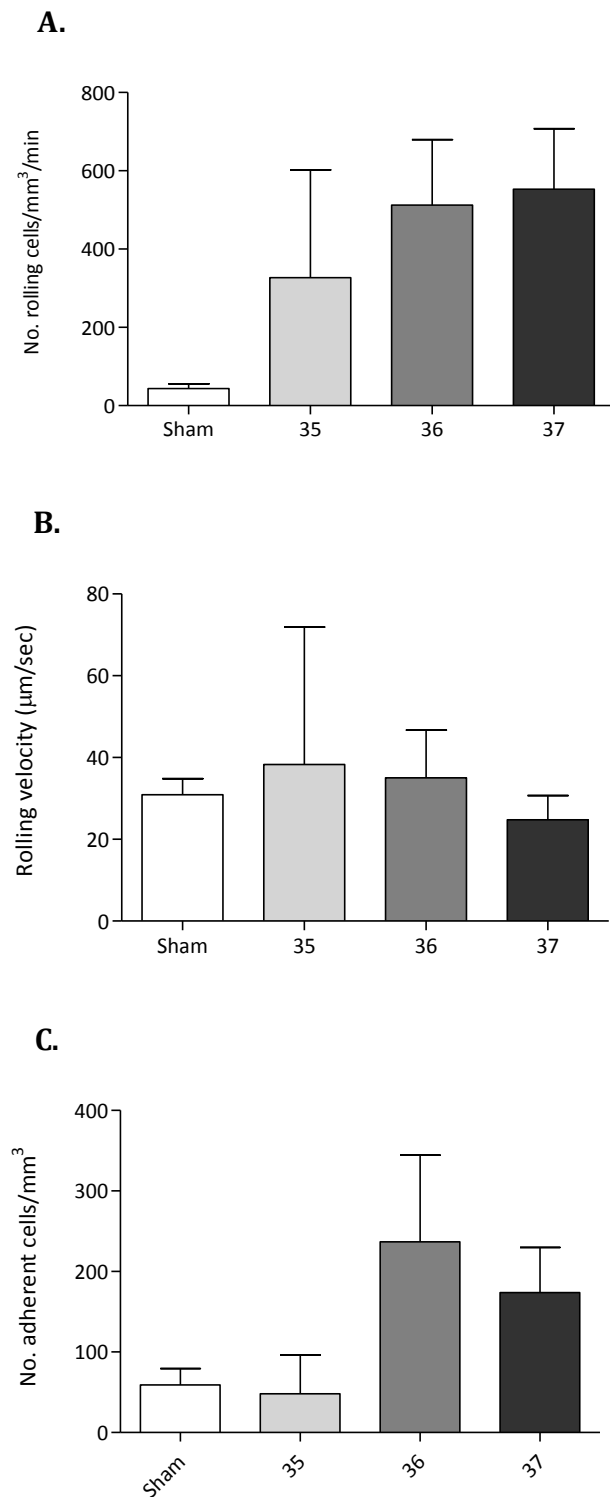


Figure 3.1: Investigation into the effect of alteration of body temperature on leukocyte-endothelial cell interactions in MCAo. Male, C57Bl6 mice (25-30g) underwent occlusion of the MCA via induction of a 210µM filament for 30min followed by withdrawal and a 24hr reperfusion time point. Leukocyte-endothelial interactions were then visualised using Rhodamine 6G and IVM. n=3-6 mice/group.

Analysis of temperature dependent infarct development and mortality rates associated with the MCAo model

Following the conduction of the IVM, the brains were removed and stained with TTC to study the development of the infarct in the ipsilateral hemisphere. Surprisingly, there appeared to be little difference in the size of the infarct that developed across the different temperature groups. Further, infarct development appeared to show a considerable amount of variability (Figure 3.2). Mortality rates associated with these altered temperatures remained high ($\approx 60\%$ at $35 + 36^{\circ}\text{C}$ and $\approx 80\%$ at 37°C) (Figure 3.3), and therefore it was investigated whether altering the ischaemia-reperfusion time points would improve survival and also data reproducibility (Figure 3.4).

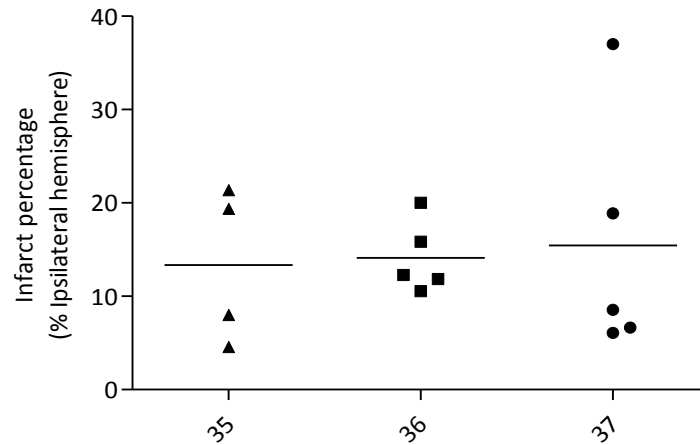


Figure 3.2: Infarct development in the ipsilateral hemisphere using MCAo following a period of 30min ischaemia and 24hr reperfusion. Male, C57Bl6 mice (25-30g) underwent occlusion of the MCA via induction of a 210 μ M diameter filament for 30min followed by withdrawal and a 24hr reperfusion time point. Following IVM, brains were taken, sliced along the coronal axis and stained in 2% TTC followed by fixing in 4%PFA. n=4-5 mice/group.

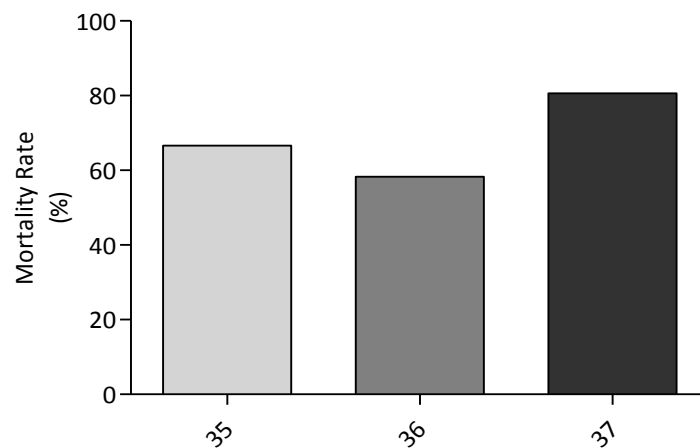
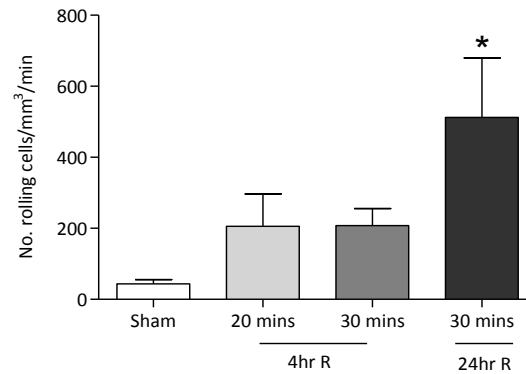


Figure 3.3: Mortality rate associated with MCAo following a period of 30min ischaemia and 24hr reperfusion. Male, C57Bl6 mice (25-30g) underwent occlusion of the MCA via induction of a 210 μ M diameter filament for 30min followed by withdrawal and a 24hr reperfusion time point. n=9-31 mice/group.

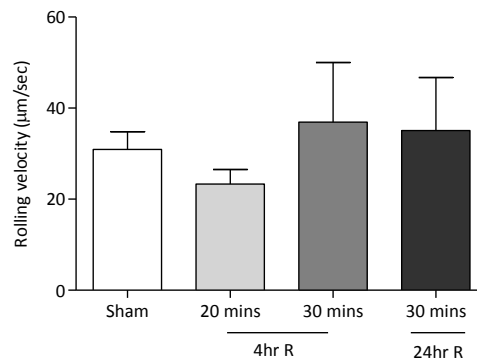
Investigation into the effect of altering the MCAo model protocol on the consistency of data generation

Reducing the time of reperfusion was shown to alter the leukocyte-endothelial interactions (Figure 3.4). The number of rolling cells was seen to markedly decrease when the reperfusion was lowered (205 (20min ischaemia), 207 (30min ischaemia) cells/mm³/min 4hr reperfusion); vs. (512 (30min ischaemia) cells/mm³/min 24hr reperfusion), although it was similar between the 20min and 30min treatment groups. Cell rolling velocity remained comparable between all groups. Cell adhesion appeared to be markedly decreased in the 30min ischaemia groups when reperfusion was decreased from 24hr to 4hr. However, paradoxically, decreasing the ischaemia time to 20min appeared to increase the number of adherent cells that were present within the microvasculature when compared to mice that had undergone 30min ischaemia with 4hr reperfusion (Figure 3.4).

A.



B.



C.

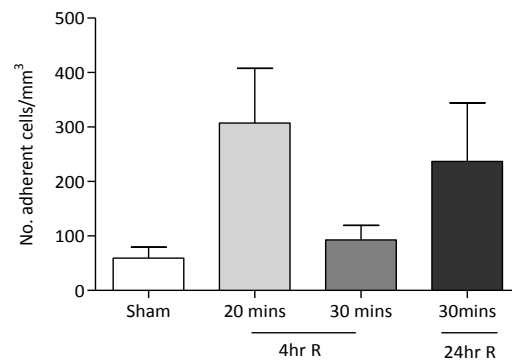


Figure 3.4: Investigation into the effect of alteration of ischaemia and reperfusion time on leukocyte-endothelial cell interactions in MCAo. Male, C57Bl6 mice (25-30g) underwent occlusion of the MCA via induction of a 210µM diameter filament for 20 or 30min whilst body temperature was maintained at 36°C followed by withdrawal of the filament and a 4hr or 24hr reperfusion time point. Leukocyte-endothelial interactions were then visualised using Rhodamine 6G and IVM. R = reperfusion. * $P < 0.05$ compared with sham treated animals using nonparametric ANOVA with Dunns post hoc test. $n = 2-4$ mice/group.

Investigation into the effect of altering filament size on the MCAo model on the consistency of data generation

It was also investigated whether alteration of the filament size would decrease the variability and improve the results gained. The number of rolling cells within the microvasculature following 30min ischaemia and 4hr reperfusion did appear to be altered in line with filament size (Figure 3.5). The smallest filament, 190 μ M diameter, was associated with the lowest number of cells rolling through the venules, which increased in line with increasing filament diameter (210 μ M and 230 μ M; Figure 3.5a). The cell rolling velocity was again relatively constant and did not seem to be affected by alterations in the filaments diameter. Following removal of the large and medium sized filament there appeared to be a relatively low number of cells adhering to the venular walls at 4hr, however, following removal of the small filament there was an increased but also significantly varied number of adherent cells.

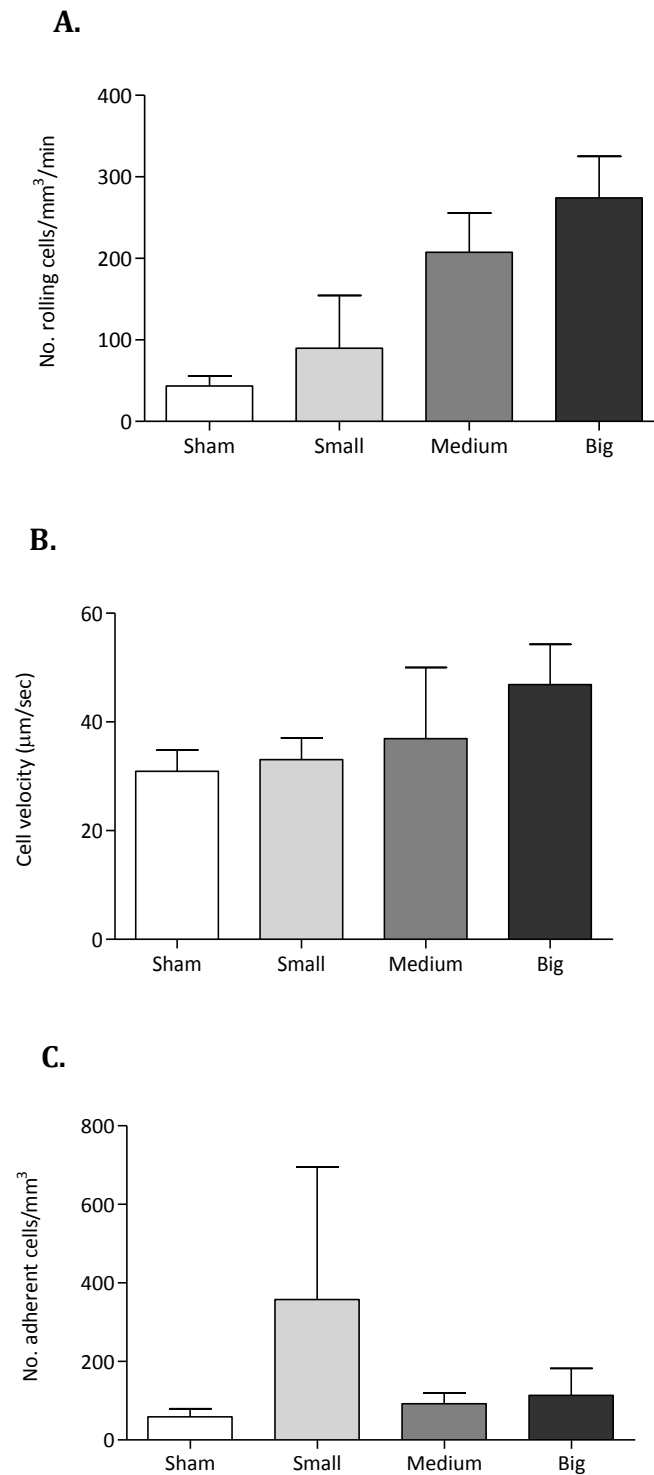


Figure 3.5: Investigation into the effect of alteration of filament size on leukocyte-endothelial cell interactions in MCAo. Male, C57Bl6 mice (25-30g) underwent occlusion of the MCA via induction of a 190 (small), 210 (medium) or 230μM (big) diameter filament for 30min whilst body temperature was maintained at 36°C. The filament was withdrawn and 4hr reperfusion time point was allowed prior to leukocyte-endothelial interactions being visualised using Rhodamine 6G and IVM. Bars represent filament size. n=2-4 mice/group.

MCAo model development discussion

The aim of using the MCAO model was to produce a reproducible model in order to investigate the leukocyte-endothelial interactions and other pathophysiological markers associated with inflammation in the post ischemic brain. As a model, MCAo represents the gold standard technique in order to investigate potential treatments in stroke therapy for several reasons. The majority of patients that enter the clinic following a stroke have undergone an ischaemic episode (~85%), and therefore the ability to recapitulate this in animal models allows investigation into: the activation of different cell types and the pathways associated with this cerebral ischaemia, the time frames necessary to improve outcome, as well as events subsequently occurring during reperfusion²⁰⁷. As the surgery involves insertion of a filament to occlude the MCA intraluminally, it does not require a craniotomy thereby minimising risk of direct damage to the brain. This means that any inflammation seen should be as a cause of vascular occlusion rather than damage that could be associated with externally clamping the MCA as may occur in other models. Mice are ideal to use for several reasons: knowledge of their genome means the availability of relevant genetically altered animals can aid hypothesis testing; the ability to compare like with like with other work on going in the group would be advantageous; smaller animals meaning lower doses and reduced reagent costs and the availability of data already published on the model in this species allows comparison of results gained with a current body of literature. However, the use of mice in this model does represent a significant surgical challenge, and due to the length of anaesthesia and post-operative immobility can be physiologically stressful to the animals. The small size of mice means that the surgery involved requires a delicate hand and significant precision. Isolation and occlusion of the vasculature involved in inserting the filament takes time to learn and requires precise and careful surgical ability.

Here, insertion of the filament into the external and common carotid artery was tested in order to ensure minimal damage to the soft tissue surrounding the vessel. Due to the vessels proximity to trachea and the fact that it underlies the salivary glands, minimal tissue disruption is important since inflammation of the area could lead to breathing difficulties and unnecessary agitation of the salivary glands leads to secretions which can affect the area. Although both vessels provided accurate filament insertion, the common carotid artery route was thought to be associated with the least tissue damage, and was therefore less likely to affect the animals' outcome (See Figure 3.6). Furthermore, through testing the optimal length of insertion advancement of the filament to 13mm from the bifurcation of the common carotid artery lead to optimal covering of the MCA (Taylor, unpublished). Also, when this position was reached slight resistance could be felt on the filament. Further advancement can lead to damage of the cerebral vasculature and surrounding tissue.

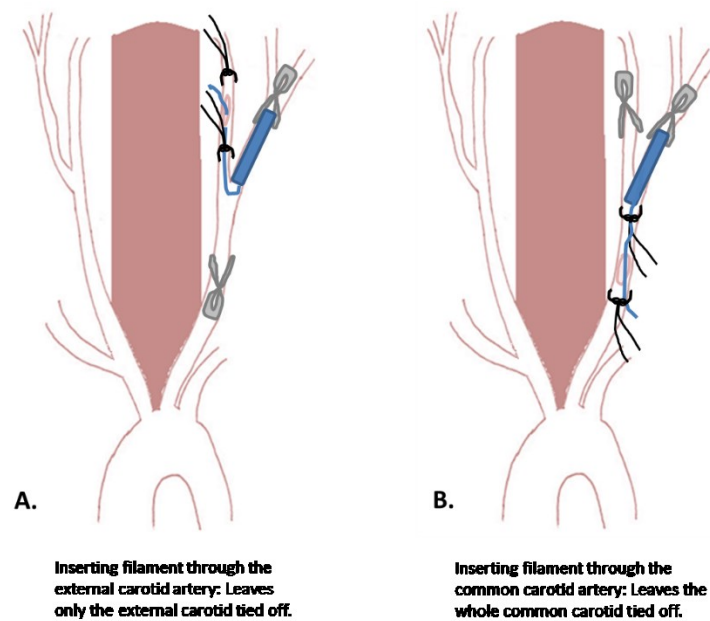


Figure 3.6: Illustration highlighting route of filament insertion. Diagrammatic representation of filament insertion into either the common carotid (A) or external carotid artery (B). (Gillespie, unpublished)

In line with this, and shown above, was the testing of various filament sizes and types in order to provide the optimal vessel occlusion. Several types of ‘homemade’ filament were tested. These included monofilaments which were cut and coated in silicone. However, the difficulty in creating uniform filaments with these methods and their durability made them unsuitable. For this reason, use of the doccol filaments provided the most suitable filament to cause the occlusion. From the results gained in Figure 3.5, as well as the mortality and infarct size associated with each filament, it was apparent that the medium filament (diameter of 210 μ m) was most suitable. The variability seen with the smaller filament (190 μ m diameter) appeared to be associated with incomplete occlusion in some animals since their TTC staining appeared to show no infarct. Both the 210 μ m and 230 μ m diameter filament were both associated with infarct development, so as the smaller filament appeared adequate this was used so as to limit damage to the vessels through insertion of a larger than necessary filament.

Whilst trying to set up the model several different anaesthetic agents and regimes were also used. Several anaesthetic agents and regimes appear to interact with the pathology associated with cerebral ischaemia-reperfusion and therefore, the anaesthetic used and the doses administered represent another parameter that must be tightly regulated when performing MCAO²⁰⁸⁻²¹⁰. Here, sodium

pentobarbital (100mg/kg) and isoflurane were tested. Sodium pentobarbital was inappropriate since its long recovery period prolonged the time the animals were immobile for and therefore presented a problem with temperature management. Isoflurane represented an attractive anaesthesia option due to its rapid recovery time and ability to control the depth of anaesthesia, however, the nature of the equipment association with the agents delivery complicated conducting the surgery and was therefore not taken forward. Furthermore, prolongation of exposure time to isoflurane has been shown to lead to significant experimental variation following ischaemic injury and was therefore not ideal for the purpose²¹⁰.

Another parameter which has been seen to affect pathophysiological outcomes following ischaemic stroke in mice is temperature. Without the correct equipment and constant monitoring temperature can be a difficult variable to accurately control. Not only do mice undergo rapid heat loss following induction of anaesthesia due to immobility and a high surface to volume ratio, their body temperature can also fluctuate markedly in the presence of external heat sources²¹¹. For these reasons, use of a homeothermic mat aimed to eliminate unwanted variation in body temperature. This also enabled investigation into the effect of altered body temperature on leukocyte-endothelial interactions, infarct development and mortality rates (Figures 3.1-3.3). Indeed, taking into account the mortality rates associated with MCAo for 30min followed by 24hr reperfusion temperature did appear to have an effect. Maintenance of the rectal temperature at 37°C appeared to be associated with a greater mortality level than that seen at either 35 or 36°C (Figure 3.1). For this reason, MCAo was no longer conducted with the rectal temperature of 37°C and 36°C was taken forward as an appropriate temperature to improve survival without decreasing temperature with a level that may be itself protective. Furthermore, the lower temperature of 35°C seemed to show a reduced level of cell adhesion compared to the other two temperature groups which could be indicative of an effect leading to altered leukocyte-endothelial cell interactions. Indeed, the use of hypothermia to dampen the inflammatory response and protect against cellular damage associated with various types of cerebral injury has received much attention²¹²⁻²¹⁴. The temperatures investigated are broad, ranging from as low as 17°C up to 35.5°C²¹³. The effects of this thermo-reduction appears to be manifold, including decreasing cellular apoptosis, altering inflammatory cytokine levels both by reducing pro-inflammatory cytokines (TNF α , IL1 and IL-6) and increasing anti-inflammatory cytokine (IL-10) levels, reducing cellular adhesion molecules and reducing ROS production^{212,215-217}.

It is also of note that there did not seem to be a correlation between increased leukocyte-endothelial interactions and the development of the infarct size (Figure 3.1-3.2). This seems somewhat surprising

since previous findings have indicated that the development of brain infarction is linked to the presence and recruitment of neutrophils to the damaged brain tissue, and that inhibiting the recruitment of these immune cells is associated with an amelioration infarct development and stroke outcome²¹⁸⁻²²⁰. These variations in temporal dynamics of cell recruitment may in part be accounted for by differences in the MCAo protocol used, the techniques used to quantify the number of cells and whether techniques were looking at the presence of intravascular, parenchymal or total immune cell numbers^{220,221}. On the other hand, the size of the infarcts that developed, although similar between groups, did show a marked variability.

Investigation into different ischaemia and reperfusion time points was explored to try and tighten the data been gained. Previously, alteration in ischaemia and reperfusion times has been shown to alter the associated presence of immune cell activation and hence, inflammatory profile present in the rodent brain^{222,223}. Variation in the leukocyte-endothelial interactions however, remained marked with a standard error of between 30-45% in the level of cell adhesion seen in some groups. This highlighted the inconsistency of the data being gained from the model at this time and brought into question the validity of its use with the time pressures associated with the project.

It was also found that when some mice had made it to the stage of drilling the cranial window in order to visualise the cerebral microvasculature, that they had increased intracranial pressure. This caused the tissue to distort at the site that the window was drilled and effect blood flow and therefore, caused difficulties in recording cellular interactions. This was potentially a combination of damage to the dura on removing the cranial window and manifestation of cerebral oedema that is known to be develop in the ipsilateral hemisphere of some mice. Breakdown of the BBB via loss of intercellular tight junction molecules such as claudin-5 and occludin, as well malfunctions in water transporters such as aquaporin 4 lead to an increase in parenchymal water content causing a subsequent elevation in intracranial pressure and potentially decreased blood flow^{224,225}. This produced a confounding factor to subsequently conduct IVM on these animals since in intracranial pressure and altered blood flow to the area caused cellular damage and bleeding to the tissue trying to be visualised.

Due to the inability to create reliable and reproducible data, and the difficulties outlined above with the MCAo model, it was decided that a model of endotoxaemia would be investigated for its applicability to visualise leukocyte-endothelial interactions within the cortical brain microvasculature. It was hoped that using this model would provide an alternative method with fewer confounding

factors in order to investigate and visualise leukocyte-endothelial interactions within the brain following an inflammatory stimulus as outlined in the aims.

Use of LPS as a model to investigate cerebral leukocyte-endothelial interactions

The use of LPS to illicit an inflammatory response, both in *in vitro* and *in vivo* experiments, represents a useful tool to study many aspects of the host immune response. The ability to refer to a known body of data regarding the use of LPS to stimulate inflammation, the simplicity of the model itself and the robust and reproducible inflammatory response LPS generates made the use of *i.p* injection of LPS (0.5mg/kg), in conjunction with IVM, an appealing model to investigate to stimulate and visualise leukocyte-endothelial cell interactions within pial venules of the cerebral microvasculature. Development of this model also allowed investigation into dose and time dependent effects of LPS on cellular interactions.

Investigation into the time dependent effects of LPS on leukocyte-endothelial interactions

Throughout the time course studied neither the sham surgery associated with the protocol, nor the vehicle treatment of saline, was associated with the presence of any marked leukocyte-endothelial interactions. There were very few cells rolling along the vessel walls, and likewise, there was an absence of cells that were adherent to the vessel wall. This was seen to be the case at the 1, 2 and 4hr time points in sham and saline treated animals (Figure 3.7). At no time point were there any significant differences in the interactions seen following either sham or saline treatment.

Similarly, following *i.p.* injection with LPS at both the 1-2hr time points there were seen to be very few cells present in the cerebral microvasculature that were either adherent or rolling along the vascular wall. However, following a 4hr incubation period with LPS there was a notable increase in cellular interactions within the venules visualised. In animals treated with LPS, the number of leukocytes rolling within venules increased significantly. Furthermore, these leukocytes exhibited a slow rolling velocity. Furthermore, there was a concomitant and significant increase in the number of leukocytes that were adherent to the vessel wall (Figure 3.7). These observations were true when LPS treatment was compared to either sham or saline treatment.

At this point it is worth noting that these effects were specific to venules. Interactions between leukocytes and endothelial cells within the arterial side of the vascular bed of the cortex were generally absent as can be seen in the still images taken from recorded videos presented in Figure 3.9.

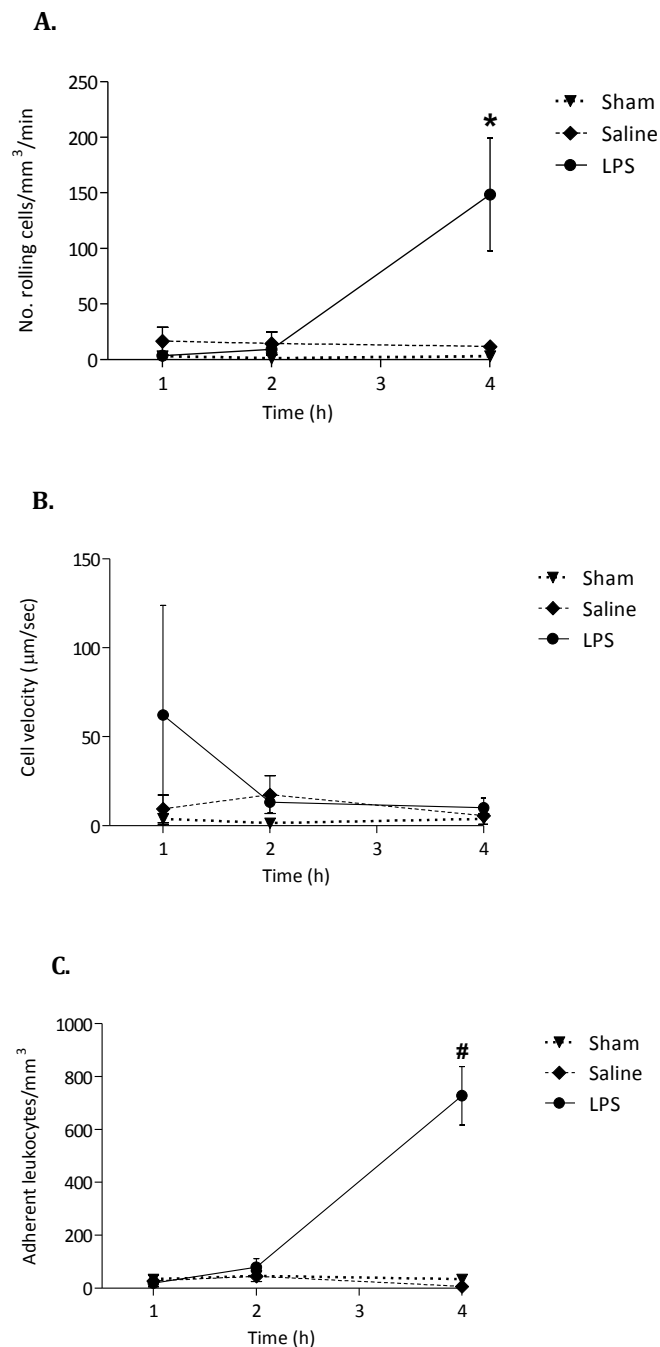


Figure 3.7: Peripheral LPS induces leukocyte-endothelial interaction in cerebral pial venules in a time dependent manner. Male, C57Bl6 mice were injected with Saline or LPS (0.5mg/kg) or underwent sham surgery, followed by visualisation of cerebral pial vessels via craniotomy at 1, 2 and 4hr post injection. A) Cell flux (per mm³/min) B) rolling velocity (µm/sec) and C) no. of adherent cell (per mm³). Data expressed as mean ± SEM. **P*<0.05 compared to sham and vehicle treated group; # *P*<0.05 compared vehicle treated group using ANOVA with Dunns post hoc test. n=4-8 mice/group

Investigation into the dose dependent effects of LPS on leukocyte-endothelial interactions

To investigate the dose dependency of these cellular interactions, a range of *i.p.* LPS doses were studied at the 4hr time point (Figure 3.8). It was apparent across all doses tested that there was an increase in cellular interactions within the parietal cortical venules following peripheral LPS.

All doses of LPS tested showed a trend towards increased numbers of cells rolling (with a significant difference seen in with doses of 0.1mg/kg and 0.5mg/kg LPS) along the venule walls in comparison to saline treated animals in which there were very few (Figure 3.8a). Similarly, the rolling velocity at which these cells were travelling in LPS treated mice was slow ranging from 5-25µm/sec (Figure 3.8b). Finally, all doses tested showed a marked increase in the number of cells that were adherent to the vessel wall of the venules within the parietal cortex, with doses of 0.1, 0.5 and 4mg/kg LPS showing significantly increased numbers of adherent leukocytes compared to saline treated animals (Figure 3.8c).

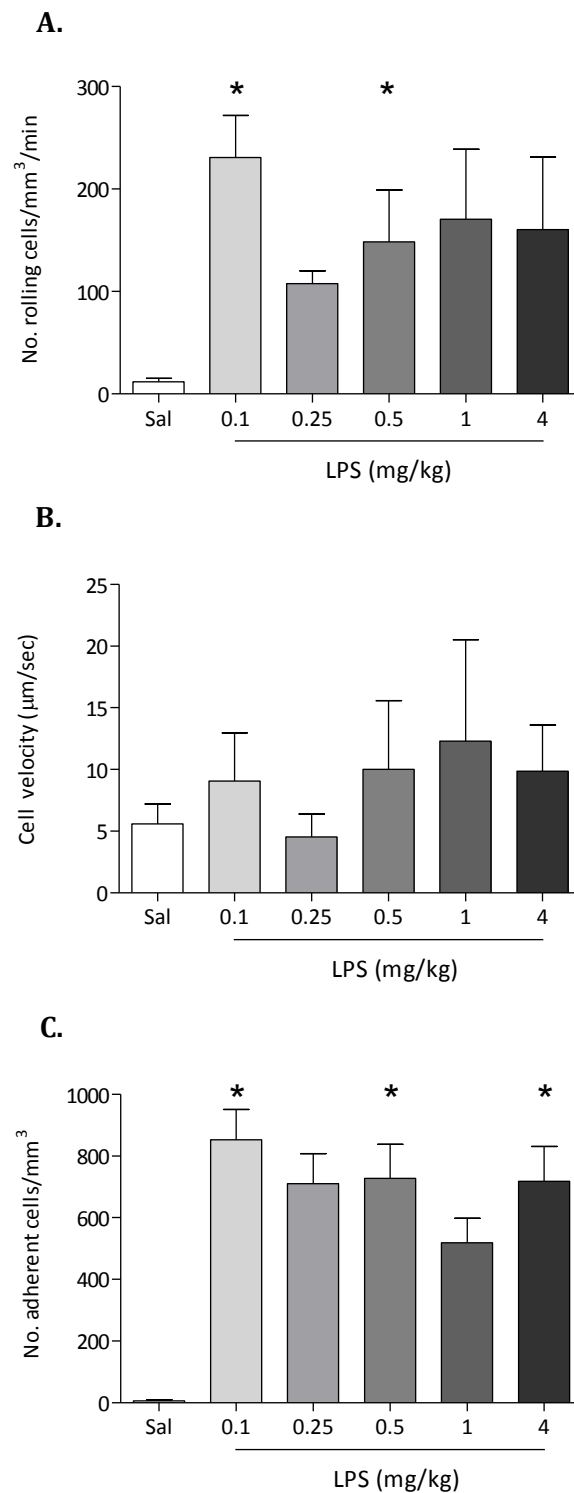


Figure 3.8: Peripheral LPS induces leukocyte-endothelial interaction in cerebral pial venules over various doses. Male, C57Bl6 mice were injected with LPS at varying doses 4hr prior to visualisation and compared to appropriate vehicles. A) Cell flux (per mm³/min) B) rolling velocity (µm/sec) and C) no. of adherent cell (per mm³). Data expressed as mean ± SEM. **P*<0.05 compared with vehicle treated group using ANOVA with Dunns post hoc test. n=3-8/group

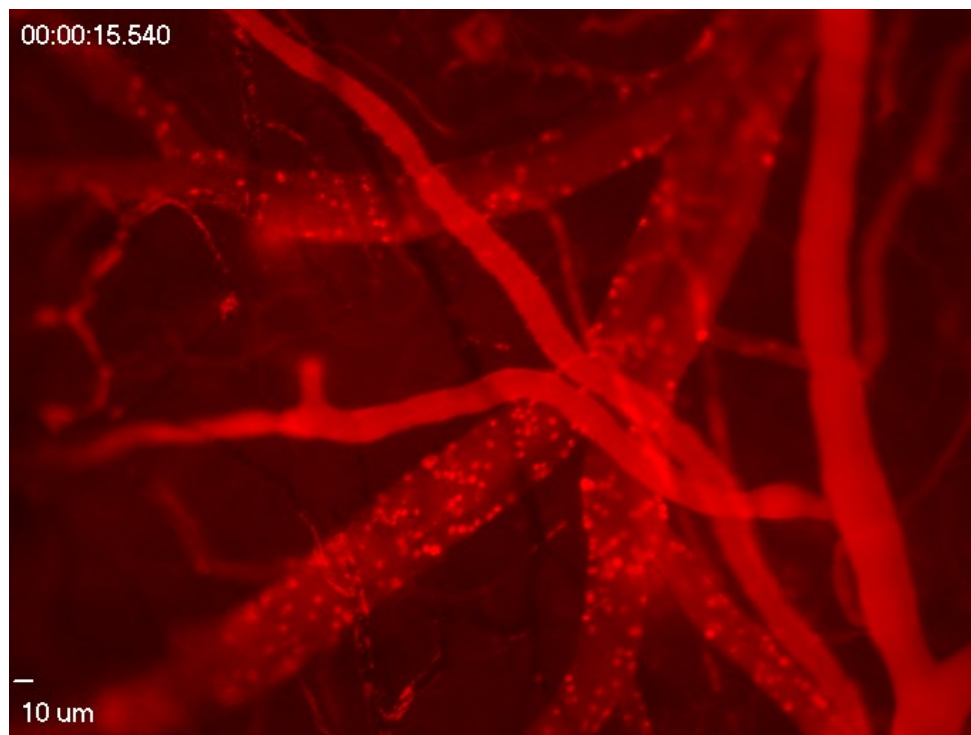
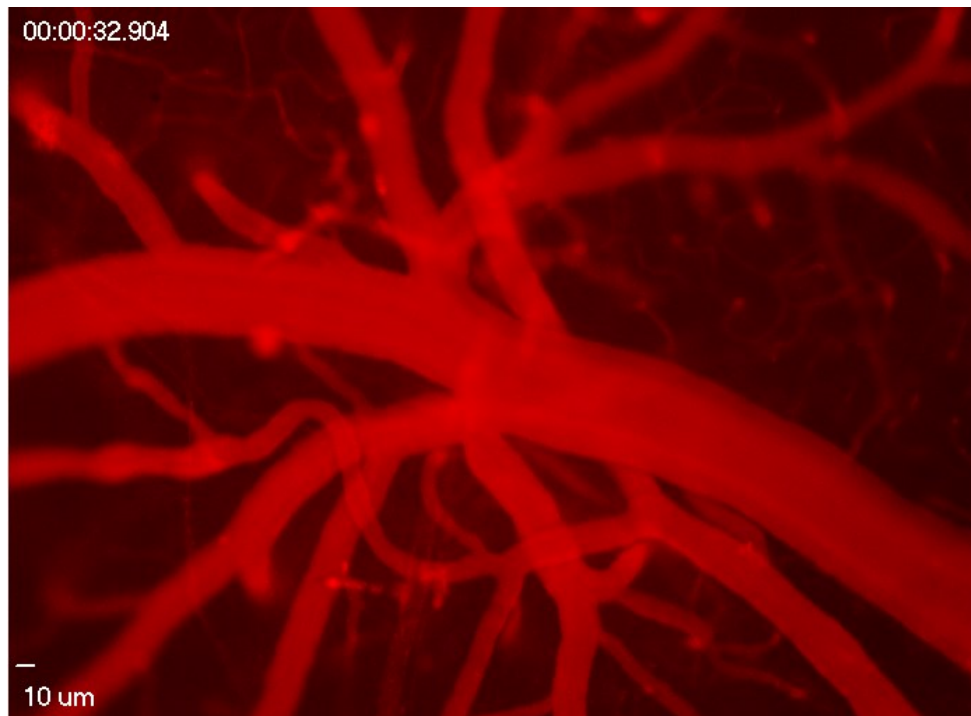


Figure 3.9: Representative photographic image of the cortical parietal microvasculature taken from mice that have undergone saline or LPS treatment respectively, showing increased presence of leukocytes specifically within the pial venules. Male, C57Bl6 mice were injected with Saline (A) or LPS, 4mg/kg (B). Parietal pial vessels were visualised after 4hr exposure. (Gillespie, *unpublished*)

LPS model discussion

The data gained using this model of endotoxaemia highlights the applicability of the endotoxaemia model as an appropriate inflammatory model which produce a consistent inflammatory response to stimulate leukocyte-endothelial interactions within the brain. Teaming this with IVM allows us to achieve one of the primary aims of the thesis.

Use of IVM

IVM used in combination with injection of fluorescent dye provides a power tool with which to study the cellular interactions induce within the brain by endotoxaemia in real time, in live anaesthetised animals. The ability to resolve individual cells means they can be tracked, and their behaviour studied, as they move along the vessel walls. Due to the depth and nature of the brain tissue, it is typically cortical vessels that are viewed, and therefore, the discussion here is in relation to cortical parietal pial vessels. Differences in flow between vessels of the arterial and venular sides of the capillary bed allow the two vessel types to be identified. Across all treatment groups there was a general absence in leukocyte-endothelial interactions within arterioles, and therefore, this data is not presented or discussed since no changes were observed. This is in support of previous data which has found that leukocyte adhesion is confined to venules except in cases of extreme vascular damage²²⁶.

The parameters investigated here allow the effect of different stimuli and drug interventions/protocols to be tested and compared. The number of rolling cells within a vessel may be indicative of upregulation of adhesion molecules involved in the early stages of the inflammatory cascade, such as P-, E- and L-Selectin; either on the endothelium or leukocytes respectively^{1,130}. When teamed with the rolling velocity this can be used as a marker for the strength of these interactions with slower rolling velocities potentially indicating the involvement of integrins and immunoglobulins which form stronger bonds, meaning they are associated with a slower rolling phenotype and therefore a decreased rolling velocity^{158,227}. Finally, increased levels of cell adhesion suggest a more inflamed environment and the likelihood for a more activated vasculature and circulating leukocytes. For this reason, IVM provides an invaluable tool with which to study the inflammatory environment within the brain and the cellular interactions it induces. In tissues other than the brain, emigration of cells out of the vasculature can be followed however, due to the thickness of the tissue this is more difficult within the brain. Development of more powerful microscopes, such as spinning disc confocal and two photon IVM's, are currently being used to aid this area of investigation.

Model of endotoxaemia

Preliminary data highlighted that peripheral injection with LPS was capable of altering leukocyte-endothelial interactions within the brain. This ability of peripherally administered LPS to induce

leukocyte-endothelial interactions within the brain has previously been reported elsewhere^{228,229}. One of the benefits of using this method of inducing a central inflammatory response in order to visualise cellular interactions is that the brain itself is not directly damaged in the procedure itself. This means that cellular interactions are a direct result of vascular and peripheral immune cell activation rather than a local immune cell activation within the brain as would be the case if intracerebroventricular injection were used.

A general absence of cellular interactions in both sham and saline treated animals highlighted that the surgery itself did not stimulate cellular interactions; likewise, neither did vehicle treatment. Firstly, these results in Figure 3.7 suggest that the surgery involved with preparing mice for use with IVM does not itself stimulate leukocyte-endothelial interactions within the cortical pial vessels, and neither does a peripheral injection *per se* (minimal interactions in saline treated animals). Secondly, the results highlight the ability of a peripheral stimulation of the immune system using LPS to induce immune cell interactions within venules of the brain microvasculature. Finally, it also highlights the temporal nature of this response in that the interactions seem to become apparent at the 4hr time point (Figure 3.7). LPS injection led to an increase in the number of cells rolling through the cortical cerebral venules (Figure 3.7a), in line with leukocytes exhibiting a slow rolling velocity. The behaviour of these cells is indicative of an increase in the expression of adhesion molecules which would support the greater interaction seen between the peripheral leukocytes and brain endothelial cells (Figure 3.7b). This is also in line with the significant increase in the number of adherent cells, which would also require an upregulation of adhesion molecules at the blood brain barrier in order for them to attach to the vascular wall (Figure 3.7c).

Indeed, the ability of LPS to stimulate leukocyte-endothelial interactions has been attributed to upregulation adhesion molecule expression and the release of pro-inflammatory cytokines from various cell types including leukocytes, platelets, endothelial cells and cells of the brain parenchyma^{228,229}. The effect of LPS on cytokine production within the plasma will be discussed further in the subsequent chapter. The adhesion molecules that have been suggested to cause these increased leukocyte-endothelial interactions are similar to those in the periphery and include molecules such as selectins, integrins and immunoglobulins (Also see Table 1.1)^{1,230}. Upregulation of the selectins on both leukocytes and endothelial cells are associated with the rolling of cells along the vessel wall and are likely to explain the increased rolling seen here.^{130,231,232}

The ability of LPS (and Sepsis) to cause haemodynamic alteration within the brain could be suggested as a factor that may alter the number of leukocytes rolling through the microvasculature²³³; however,

other groups using a similar protocol to that used here, and studying blood flow in the periphery, found that at this dose and time point LPS injection did not appear to be associated with altered haemodynamics based on shear rate measurements²³⁴. Measurement of alterations in cerebral blood flow using technology such as a laser Doppler flow would be beneficial to investigate this in order to confidently rule out this effect. This was not something available at the time these experiments were conducted; although, previous studies within the group found shear stress within the cerebral microvasculature was not affected at this dose of LPS (Gavins, *unpublished*).

LPS has previously been shown to release leukocyte haemopoietic bone marrow stores which could contribute to the increased cell flux²³⁵. Peripheral cell counts did suggest that LPS injection was associated with a significant increase in the number of circulating neutrophil and lymphocyte numbers (Table 4.1). Peripheral cell counts did highlight the presence of both neutrophils and lymphocytes in the peripheral blood, and interestingly, previous reports have suggested that 90% of the leukocytes interacting with the BBB following peripheral LPS injection were postulated to be neutrophils²²⁸.

In addition, these effects of LPS to induce leukocyte-endothelial interactions have been suggested to occur due to activation of TLR4 on the endothelium²³⁶. Although this is not something to have been investigated here, it is an interesting phenomenon and may be of consequence later in the discussion of the effects seen with SFN. Furthermore, this complementary data suggests that, at least at the time point studied here, microglial activation does not appear to play a role in driving these interactions²²⁸. This finding potentially places the onus on a more peripheral immune cell and endothelial driven response to LPS in this model of endotoxaemia and would therefore be of interest to take into account when investigating SFN's potential effects.

The consistency in leukocyte-endothelial interactions seen across the doses of LPS tested here (Figure 3.8) may be supported by the findings of Granger *et al* (*unpublished*) that the expression of E- and P-selectin are upregulated within the brain across a broad range of LPS doses (3-50µg/mouse) at 3-4hr respectively. Knowing that the expression of these selectins aids the rolling of leukocytes along the endothelial surface supports the effects seen here. This group have also published work showing the upregulation of ICAM-1 expression in response to LPS within the brain, and further, highlight the sensitivity of the brain tissue in particular to the effects of LPS in this response²³². This study did not report dose dependent effects of LPS on ICAM-1 expression but it does highlight the sensitivity of the brain tissue to respond to peripheral immune challenge. Equally, the ability of LPS to induce TNFα and IFNγ is likely to play a role in the upregulation of these adhesion molecules; marked increases in both

these cytokines within the plasma were seen here (Table 4.1) and the circulation of these would support and/or enhance the increased expression of adhesion molecules reported by these pro-inflammatory mediators elsewhere^{231,232}.

Discussion on appropriate use of model of cerebral inflammation

The primary aim of this Chapter was to validate an appropriate model of cerebral inflammation in order to investigate the leukocyte-endothelial interactions that underlie this physiological response to immune stimulation within the brain microvasculature.

Several obstacles were faced when investigating the use of the MCAo model. This model was initially chosen due to its ability to reproduce pathological pathways activated following ischaemic cerebral infarction in the human stroke condition. The aim was that use of this model would allow investigation into leukocyte-endothelial interactions over specific periods of tissue reperfusion, and link this to the development of cerebral tissue infarct development and other markers of inflammation such as cytokine release and alteration of protein expression. However, due to the high mortality rates experienced with the model and the inconsistency of the data gained continued use of this model was not appropriate.

Therefore, the use of LPS as a stimulant of vascular inflammation was pursued. Although the mechanisms underlying the inflammatory response initiated through the use of this inflammogen are different to that associated with MCAo; for example,

- The inflammation is driven primarily by immune cells of the periphery and vasculature
- There is no direct cerebral ischaemia response
- There has been no reported cerebral cell death and infarction

However, the inflammatory pathways do overlap in some aspects, i.e. activation of TLR4 on immune cells leading to inflammatory cell signalling, immune cell activation and cytokine induction. Furthermore, there are benefits to the use of LPS as a model of inflammation in that the technique used for its induction is much more simple, requires much less intervention and therefore is a lesser source of variation. Also, the ability to recapitulate aspects of the model *in vitro* on single cell populations is an attractive tool for dissecting mechanisms of actions and investigating cell specific functional effects.

Most importantly, the aim of this Chapter was to generate a model that induced reproducible leukocyte-endothelial interactions within the cerebral parietal cortex that could be visualised using IVM technology in order to access leukocyte-endothelial interactions, and the data generated here highlight that use of LPS in order to do this and the suitability of IVM and Rhodamine to visualise these interactions via cranial window.

Chapter 4:

INVESTIGATION INTO LEUKOCYTE- ENDOTHELIAL INTERACTIONS IN THE CORTICAL BRAIN MICROVASCULATURE DURING ENDOTOXAEMIA AND PROTECTION AFFORDED BY SFN

Outline

Work in the previous Chapter highlighted the applicability of LPS to stimulate leukocyte-endothelial interactions with the brain. The work also highlighted the usefulness of IVM as a sensitive imaging modality with which to visualise these interactions. Together the use of LPS and IVM to generate an *in vivo* model to visualise cerebral inflammation provided the ability to study the effects of SFN on the leukocyte-endothelial interactions during a cerebrovasculature response. The rationale in visualising these effects being that, it is known that leukocyte-endothelial interactions (as depicted in Figure 1.3) are a hallmark of the evolving inflammatory response; therefore, an agent that could alter these interactions may have potential anti-inflammatory effects, and highlight its potential for use as a therapeutic agent in cerebrovascular pathologies.

Aims:

Therefore the preliminary aim of this Chapter was to:

1. Use the LPS model outlined in the previous Chapter to investigate the ability of SFN to alter the leukocyte endothelial interactions during the vascular cerebral inflammatory response

To gain further understanding into how SFN may affect the inflammatory response associated with LPS, collection of plasma through cardiac puncture blood sampling was used to investigate the systemic cytokine response associated with the LPS and SFN treatment protocol. Peripheral cell counts were also used to see how leukocyte and platelet cell numbers are affected by the treatment protocol, and see how these changes may be associated with any alterations in cellular interactions noted using IVM.

The secondary aim of this Chapter which was to:

1. Use molecular *in vitro* approaches to study how SFN alters protein expression in brain tissue and cytokine production in response to inflammatory challenges

Collection of cortical brain tissue was used to investigate whether any effects associated with SFN treatment led to physiological alteration of Nrf2, owing to the previously published effects of SFN on Nrf2 previously noted and discussed in Chapter 1^{237,238}. Use of an *in vitro* neutrophil assay allowed the specific effect of SFN on this leukocyte population to be studied to understand whether a specific effect on this cell type may be associated with outcomes from the *in vivo* model.

4.1. Results

Effect of SFN pre-treatment (saline vehicle) on leukocyte-endothelial interactions stimulated via LPS

The first set of experiments in this Chapter investigated the use of pre-treatment with SFN (dissolved in saline) to alter leukocyte-endothelial cellular interactions. Saline vehicle was chosen, as previously studies investigating SFN's anti-inflammatory effects had highlighted positive findings with this vehicle and the thought that this vehicle would not interfere with inflammatory effects being visualised in the model made it an attractive choice of vehicle⁴⁰.

Saline pre-treatment did seem to alter the number of cells rolling through the microvasculature, showing a significant increase in Sal/LPS treated mice when compared to LPS treatment alone or Sal/Sal treatment. The rolling velocity of these cells was not significantly altered in any treatment protocol (Figure 4.1a/b).

When looking at the level of cell adhesion, all LPS treated mice were seen to have increased numbers of adherent cells within the pial vessels visualised. These increased levels of cell adhesion were only significant from the controls in mice treated with LPS alone (Figure 4.1c). SFN at the 3 doses tested in this treatment protocol (using saline as a vehicle) did not result in a significant alteration in either the number, or speed of cells rolling through the pial venules; neither did it significantly change the number of adherent cells counted within the vessels (Figure 4.1).

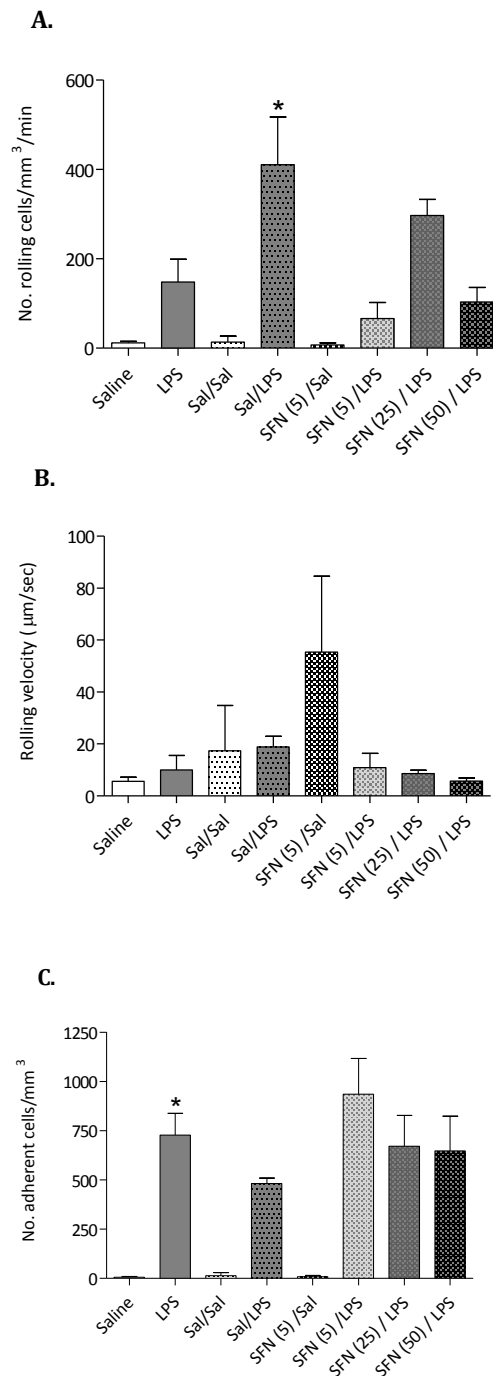


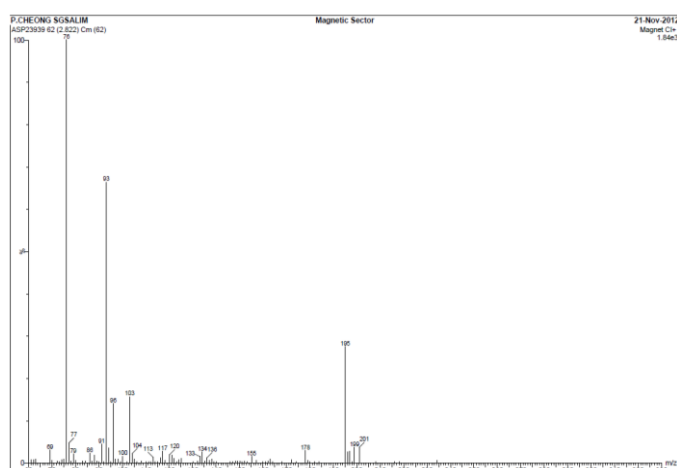
Figure 4.1: SFN in saline vehicle alters LPS induces leukocyte-endothelial interaction in cerebral pial venules but does not decrease cell adherence. Male, C57Bl6 mice were injected treated with increasing doses of SFN at 5, 25 or 50mg/kg (or vehicle saline) for 24hr prior to treatment with LPS (0.5mg/kg) for 4hr. Cerebral pial vessels were visualised and compared to appropriate vehicles. A) Cell flux (per mm³/min) B) rolling velocity (μm/sec) and C) no. of adherent cell (per mm³). Data expressed as mean ± SEM. **P*<0.05 compared with vehicle treated group using ANOVA with Dunns post hoc test. n=3-8/group

Investigation into the stability of SFN in various vehicles using mass spectrometry

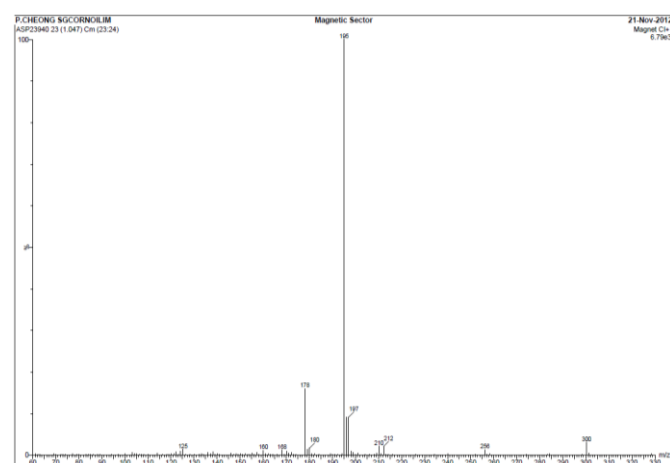
SFN has proven anti-inflammatory effects in a plethora of diseases, and therefore, these findings (Figure 4.1) appeared to contradict a large body of previously published work^{25,239,240}. For this reason, mass spectrometry was undertaken on samples of SFN that had been stored in various vehicles at -20°C for one month in order to check their stability. Chemical ionisation with Cl^+ was carried out and the resultant spectra are displayed. Figure 4.2 shows the mass spectra for samples that had been stored in saline, corn oil or a corn oil/PBS emulsion. Figure 4.2a shows SFN which had been dissolved in saline, with the expected peak representing SFN at 195m/z (SFN's molecular weight (177.29) + Cl^+ (18)). However, there also seems to be multiple other ionisation peaks suggestive of presence of several breakdown products. On the other hand, Figure 4.2b and c show much cleaner spectra with the main peak at 195m/z representative of ionised SFN conjugated to Cl^+ with fewer surrounding peaks.

For this reason, the protocol used in Figure 4.1 (SFN dissolved in saline) was repeated using a more suitable vehicle for SFN (corn oil/PBS) with the hope that this improved stability, and therefore, would allow a more conclusive investigation into whether SFN could affect leukocyte-endothelial interactions in the LPS model earlier validated.

A.



B.



C.

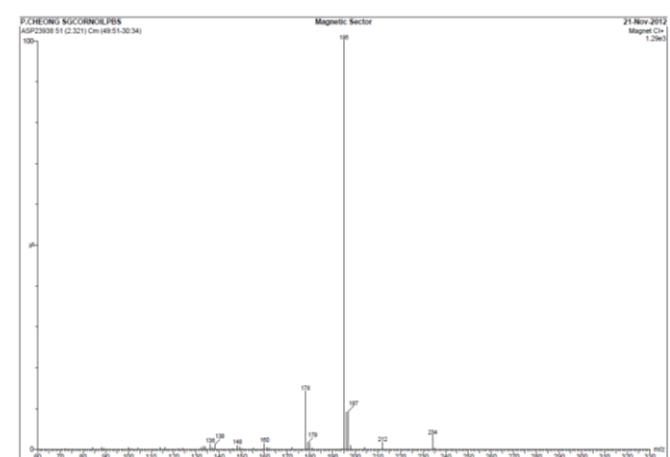


Figure 4.2: Mass spectra (chemical ionisation) showing the ionised SFN peaks $[M+NH_4]^+ m/z$ 195 following dilution in various vehicles. SFN was diluted in saline (A), pure corn oil (B) or corn oil/PBS (C) as for the preparation for animal injections. Samples were then aliquoted out and sent for mass spectrometry analysis with chemical ionisation in order to observe compound stability.

Effect of SFN corn oil/PBS treatment on leukocyte-endothelial interactions stimulated via LPS

Following on from the highlighted much improved stability of SFN in corn oil, SFN pre-treatments were re-run using corn oil and PBS as a vehicle. To ensure that the pre-treatment of corn oil would not affect subsequent LPS responses, this vehicle was tested against LPS alone (Figure 4.3). Corn oil treatment did not seem to affect the number of cells that were present within the microvasculature, or how they were interacting with the vessel wall. Responses to saline or LPS injection showed similar levels of cells rolling through the pial venules and the speed at which they were doing so regardless of whether animals had been pre-treated with corn oil and PBS or not (Figure 4.3a and b). Likewise, similar numbers of cells were adherent to the pial vessel wall following pre-treatment with corn oil/PBS as in those that had received not pre-treatment (Figure 4.3c). Importantly, there remained a significant difference in the amount of cells that were rolling and adherent following LPS treatment whether corn oil was present or not (Figure 4.3a and c). Therefore, corn oil represented a suitable vehicle for SFN dilution as it maintained the molecular stability and that it did not affect the subsequent leukocyte-endothelial interactions induced by LPS within the cerebral parietal cortex. Therefore, this vehicle was used to investigate whether SFN could affect the cellular interactions downstream of LPS injection.

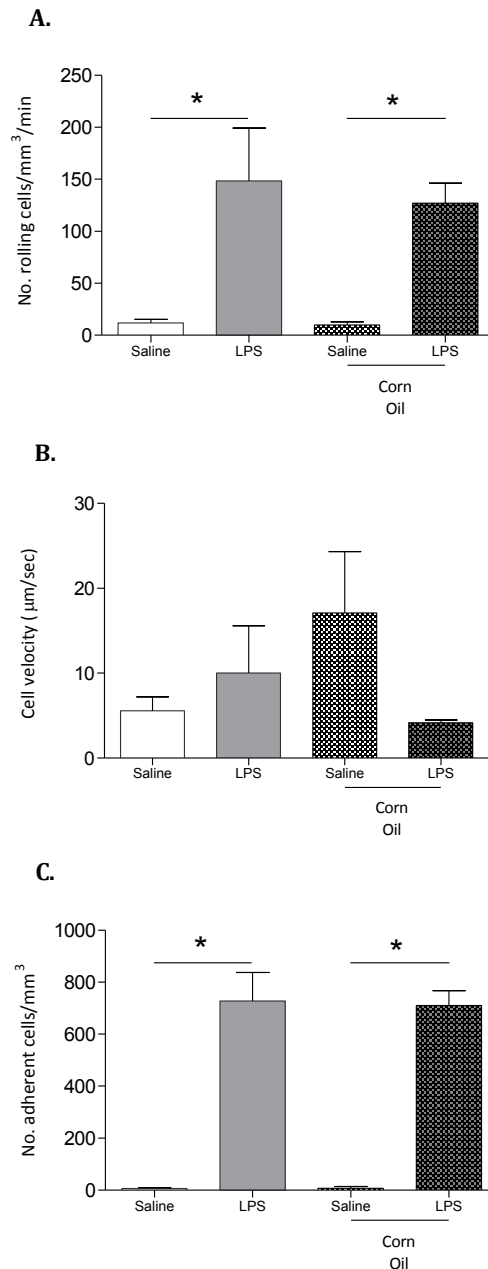


Figure 4.3: Corn oil vehicle does not alter LPS induced leukocyte rolling and adherence in cerebral pial venules. Male, C57Bl6 mice were injected with saline or LPS (0.5mg/kg), with subsequent groups receiving a prior administration of corn oil/PBS 24hr prior to treatment with LPS (0.5mg/kg) for 4hr. Cerebral pial vessels were visualised and leukocyte-endothelial cell interactions quantified. A) Cell flux (per mm³/min) B) rolling velocity (µm/sec) and C) no. of adherent cell (per mm³). Data expressed as mean ± SEM. **P*<0.05 when compared to indicated group using ANOVA with Dunns post hoc test. n=6-8/group

Effect of SFN pre-treatment (corn oil/PBS vehicle) on leukocyte-endothelial interactions stimulated via LPS

As mentioned previously, the cellular interactions stimulated by LPS injection were not significantly altered in the presence of corn oil pre-treatment, and there continued to be an increase in cell rolling and adhesion associated with LPS injection as previously shown and discussed (Figure 4.3).

SFN treatment at 5mg/kg did not appear to be effective at decreasing leukocyte-endothelial interactions. There was no apparent difference between mice that had received corn oil pre-treatment or SFN treatment (5mg/kg) on the number of rolling cells (Figure 4.4a). Likewise, there was no significant alteration in the cell rolling velocity or in the number of adherent cells in SFN 5mg/kg treated mice in comparison to those pre-treated with corn oil (Figure 4.4b and c).

When the dose of SFN was increased to 50mg/kg, however, SFN treatment was associated with alterations in leukocyte-endothelial interactions within the cerebral microvasculature. Firstly, SFN (50mg/kg) was associated with a marked increase in the number of cells rolling through the microvasculature. This increase in the number of cells rolling through the pial venules was not however significant when compared to mice that had received either corn oil/LPS or SFN at 5mg/kg with LPS treatment (Figure 4.4a). The speed at which these cells were rolling in SFN(50mg/kg)/LPS treated mice was significantly increased when compared to mice that had received corn oil pre-treatment and LPS.

Treatment with SFN (50mg/kg) was also associated with a marked decrease in the number of cells that were adherent within the cerebral microvasculature with numbers decreasing to more than half that seen in animals treated with corn oil and LPS (711 vs. 334 cells per mm³). These changes were not significant, however, mice receiving this dose of SFN no longer showed a significant difference in the number of adherent cells when compared to animals treated with corn oil and saline or SFN (50mg/kg) and saline (Figure 4.4c).

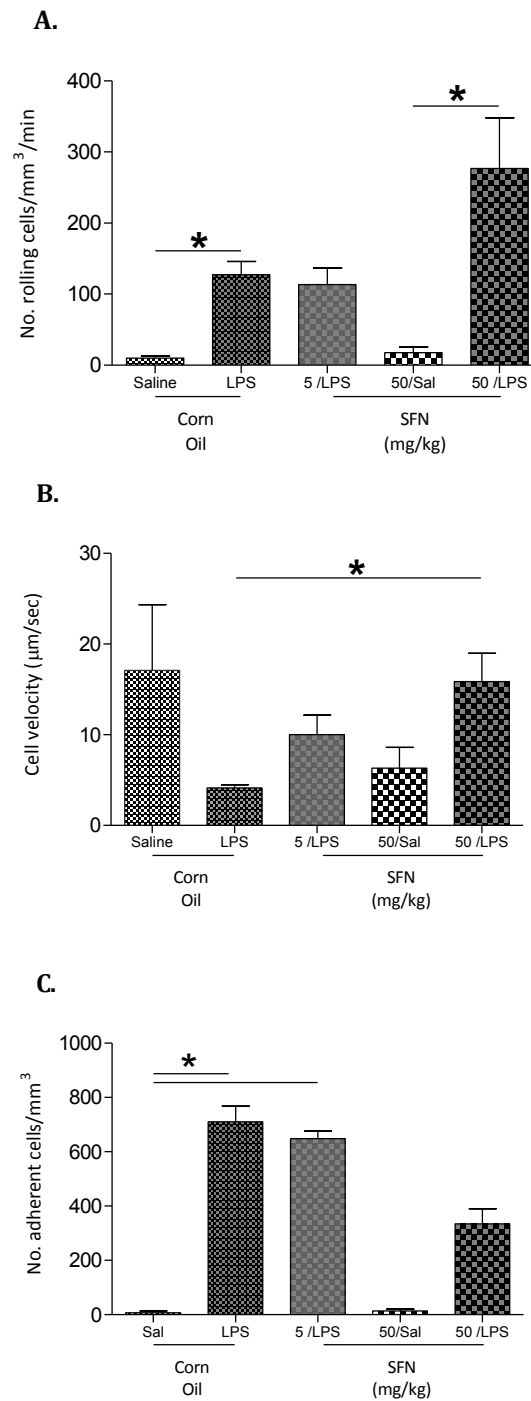


Figure 4.4: SFN in corn oil vehicle alters LPS induces leukocyte rolling and adherence in cerebral pial venules. Male, C57Bl6 mice were injected with saline or LPS (0.5mg/kg), with subsequent groups receiving a prior administration of corn oil/PBS or SFN(mg/kg) at indicated doses 24hr prior to treatment with LPS (0.5mg/kg) for 4hr. Cerebral pial vessels were visualised and leukocyte-endothelial cell interactions quantified. A) Cell flux (per mm³/min) B) rolling velocity (µm/sec) and C) no. of adherent cell (per mm³). Data expressed as mean ± SEM. **P*<0.05 when compared to indicated group using ANOVA with Dunns post hoc test. n=6-8mice/group

Effect of SFN pre-treatment (corn oil/PBS vehicle) on peripheral immune cell counts

Peripheral cell counts taken via blood sampled via cardiac puncture were somewhat variable (Table 4.1). There was a trend towards increased neutrophils following treatment with LPS in naïve animals, as well as those receiving corn oil or SFN. Differences between neutrophil counts following corn oil or SFN were absent and less marked respectively. Monocyte levels appeared to be effected to a lesser level by the treatment regimen and no significant decrease across the groups were seen. Circulating numbers of these cell were much lower than any other leukocyte population. Lymphocyte levels appeared to be lower in all LPS treated animals when compared to their saline pre-treated counterpart. Both corn oil and SFN appeared to further enhance this difference by appearing to increase peripheral lymphocyte counts, with a significant difference between the lymphocyte levels of saline and LPS treated animals being apparent following SFN treatment. Platelet levels were relatively comparable across groups with no significant alterations seen, yet, elevated platelet counts did appear to be present in mice receiving SFN (50mg/kg) and saline.

Cell type	Treatment					
	Sal	LPS	Corn Oil		SFN (50mg/kg)	
			Saline	LPS	Saline	LPS
Neutrophils	9.9 ± 7.9	36.7 ± 15.7	33.7 ± 21.1	32.7 ± 17.7	42.7 ± 17.1	35.7 ± 27.7
Monocytes	5.7 ± 3.0	7.5 ± 6.1	8.3 ± 2.6	3.3 ± 4.1	3.3 ± 4.1	2.5 ± 4.2
Lymphocytes	133.6 ± 48.3	65.5 ± 29.5	241.7 ± 115.5	61.7 ± 9.3	438.3 ± 142.9	67.5 * ± 51.6
Platelets	107900 ± 36514	93133 ± 5446	100500 ± 24558	89750 ± 20349	232233 ± 303128	98517 ± 30085

Table 4.1: Differential peripheral cell counts. Blood was collected, 4hr post LPS treatment with or without prior SFN treatment (-24hr, 50mg/kg), via cardiac puncture. Leukocytes counts were performed using Turks solution (1:20), and platelet counts were performed in buffered ammonium oxide (1:100 dilution). Values represent mean cell count (x10⁴) per ml ± SD. *P<0.05 when comparing SFN/Sal and SFN/LPS treated groups using ANOVA with Dunns post hoc test. n = 6 mice/treatment group.

Analysis of Nrf2 subcellular localisation following LPS/SFN treatment protocol

Nuclear fractions from cortical brain samples were isolated using dissection of the cortex and cell fractionation. Nuclear subcellular fractions were then quantified using an ELISA. There appeared to be no significant difference in the nuclear localisation of Nrf2 in any treatment group regardless of whether mice had received treatment with LPS or SFN (Figure 4.5).

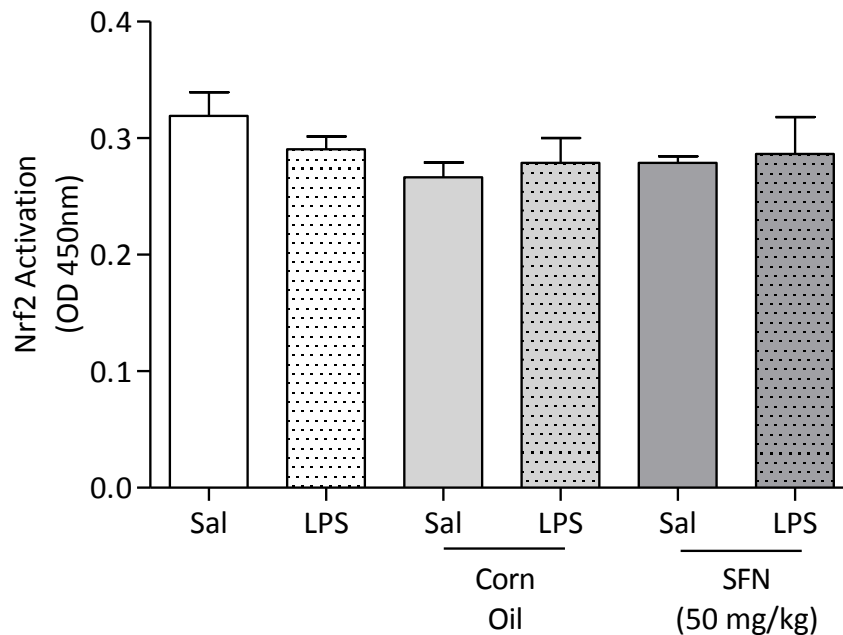


Figure 4.5: Nuclear Nrf2 Expression does not vary across treatment groups. Brains were collected from mice receiving Saline or LPS (+4hr, 0.5mg/kg) with or without subsequent corn oil or SFN(-24hr; 50mg/kg) pre-treatment, and the cortices were dissected for Nrf2 expression quantification via ELISA, Values represent mean optical density $\pm$ SD. n = 3 mice/treatment group

Effect of LPS/SFN treatment protocol on peripheral cytokine profile

Blood collected from mice that had been treated with the LPS/SFN treatment protocol was used in order to quantify the effects of SFN on peripheral cytokine expression (Figure 4.9 and 4.10). IL12-p70 appeared to be present at low levels across all treatment groups with detection in the plasma not reaching above 10pg/ml in any treatment group; there need not appear to a significant changes between any treatment group at the time point tested ((SFN -24hr, LPS +4hr) Figure 4.6a). Across all other cytokines tested, there appeared to be undetectable circulating cytokines in the saline treated animal, regardless of the pre-treatment they received. Circulating TNF α levels was increased in all animals receiving LPS, and these effects were not altered significantly by either corn oil or SFN pre-treatment (Figure 4.6b). Likewise, MCP-1 and IL-6 were both evidently increased following LPS treatment (with nearly all of the animals MCP-1 levels exceeding the maximum detectable level of the kit), and from appeared to be largely unaffected by prior corn oil or SFN pre-treatment (Figure 4.7a and c respectively). IFN γ was again increased following administration of LPS in groups tested, and there did appear to be a trend towards decreased levels in animals receiving SFN pre-treatment (Figure 4.6c). IL-10 levels were also increased by LPS treatment, and although there appeared to be some variability between the groups, levels did not appear to correlate with any particular pre-treatment protocol (Figure 4.7b).

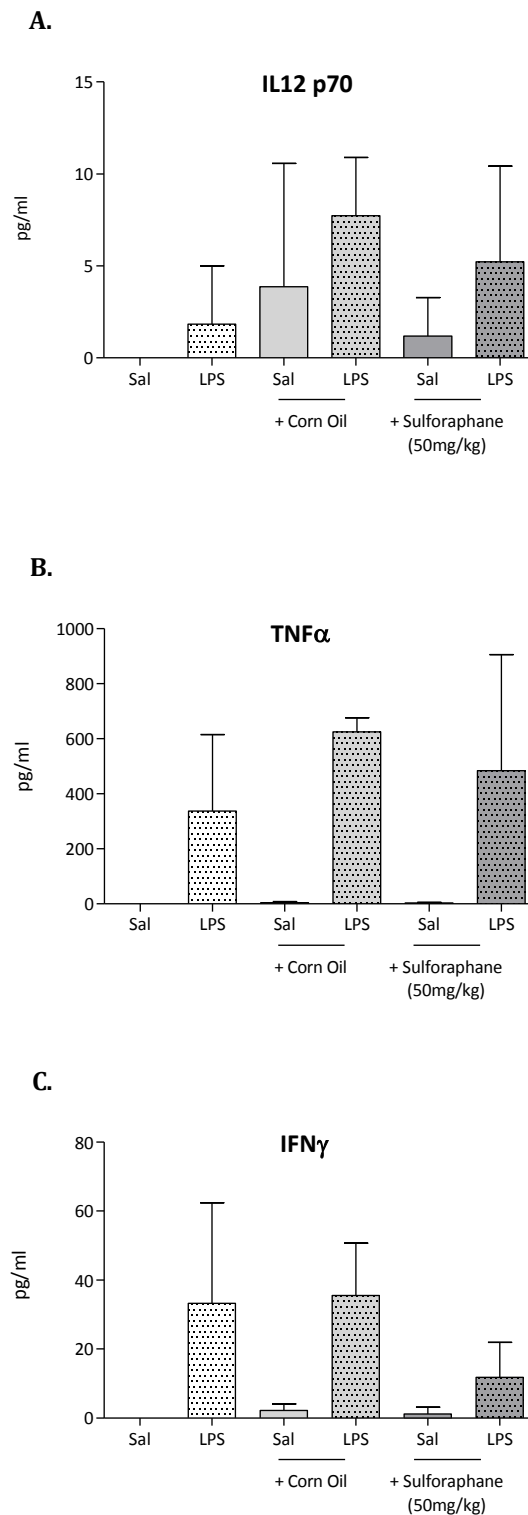


Figure 4.6: Plasma expression of IL12-p70, TNF α and IFN γ following SFN and LPS treatment protocol. Plasma was collected via cardiac puncture from mice receiving Saline or LPS (+4hr, 0.5mg/kg) with or without pre-treatment with corn oil or SFN(-24hr; 50mg/kg) via FACS cytometric BEAD array. Values represent means $\pm$ SD. n = 3 murine plasma samples/treatment group

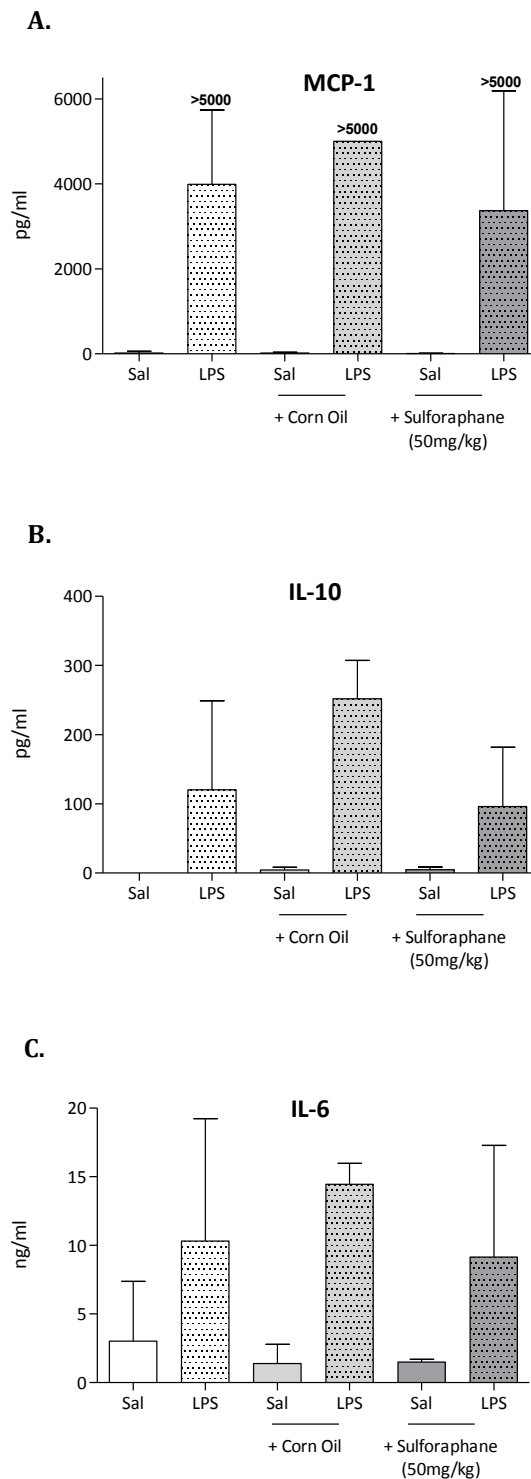


Figure 4.7: Plasma expression of MCP-1, IL-10 and IL-6 following SFN and LPS treatment protocol. Plasma was collected via cardiac puncture from mice receiving Saline or LPS (+4hr, 0.5mg/kg) with or without pre-treatment with corn oil or SFN(-24hr; 50mg/kg) via FACS cytometric BEAD array. Values represent means $\pm$ SD. <5000 indicates animals within this group had MCP-1 levels beyond the maximum detectable range. n = 3 murine plasma samples/treatment group

LPS stimulated IL-1 β production as a marker of functional neutrophil alteration following SFN and associated LDH release

To further support the work in this Chapter, a functional assay using isolated human neutrophils was conducted in order to study the effects of LPS and SFN on this cell type specifically (Figure 4.8). It was found that neither the protocol itself, nor any of the doses of SFN tested were associated with any detectable induction of IL-1 β release. However, treatment with LPS (1 μ g/ml) was associated with an increased level of IL-1 β release ($\approx$ 20pg/ml). Treatment with the doses of SFN tested here no did seem to affect this IL-1 β release at this time point. Equally, none of the treatment groups tested in this assay were associated with induction of LDH release as a marker of cytotoxicity (Figure 4.9).

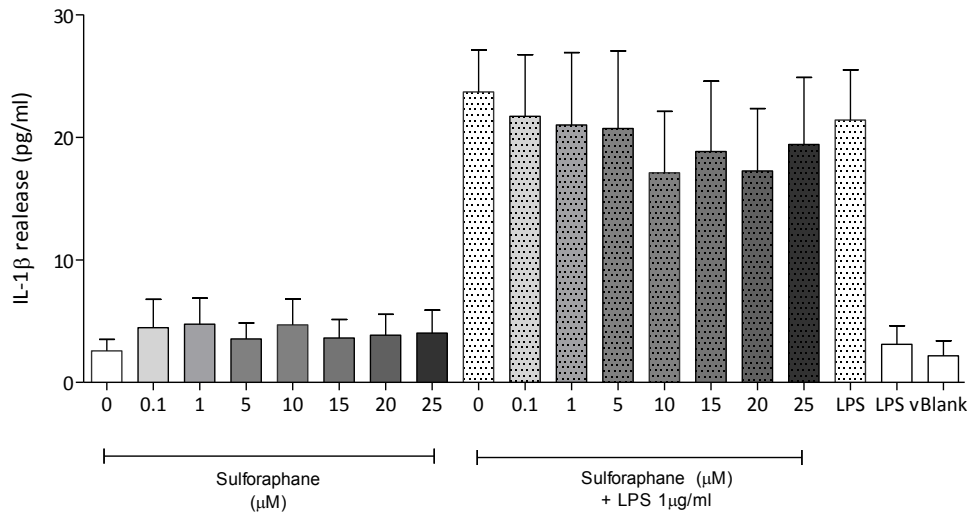


Figure 4.8: SFN administration is not associated with alterations in release of IL-1 β following treatment with LPS. Isolated neutrophils were resuspended in RPMI with glutamine (2mM), streptomycin (100Units) and FCS (10%) at 5.5×10^4 cells/ml, and incubated with SFN over various doses for 4hr prior to 14hr treatment with LPS (1 μ g/ml) min. Data expressed as mean IL-1 β release $\pm$ SEM. n=4 donors/group

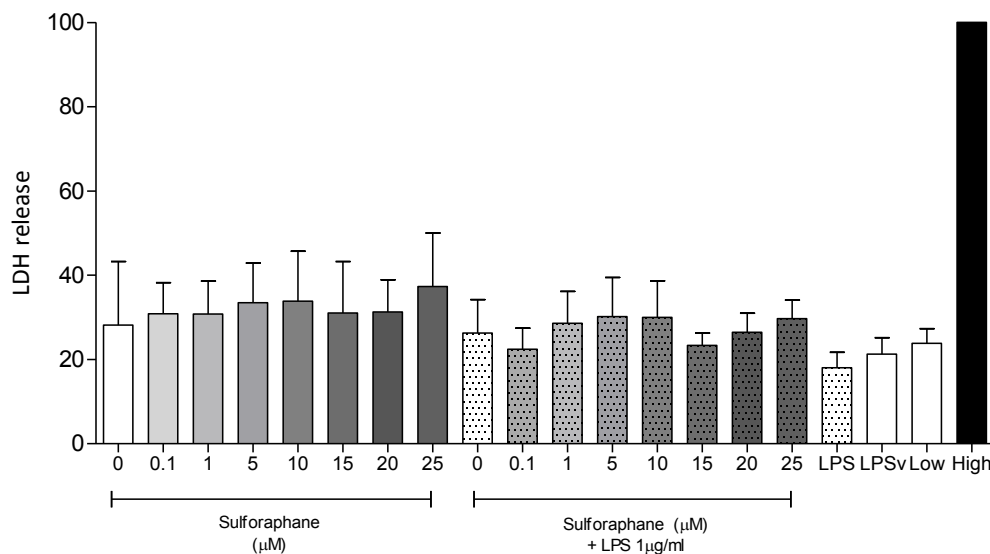


Figure 4.9: SFN treatment is not associated with increases in LDH release associated with the neutrophil stimulation assay. Isolated neutrophils were resuspended in RPMI with glutamine (2mM), streptomycin (100Units) and BSA (5%) at 5×10^4 cells/ml, and incubated with SFN over various doses for 4hr prior to 14hr treatment with LPS (1 μ g/ml) min. Data expressed as a mean percentage of LDH released stimulated by complete cell lysis $\pm$ SEM. n=3 donors/group

4.2. Discussion

Finding the right vehicle for SFN

Following on from the characterisation of the leukocyte-endothelial interactions stimulated by LPS, the aim of this Chapter was to investigate whether SFN could alter these cellular interactions, and if so, whether this would be associated with any alteration of the function of inflammatory cells. Existing literature had shown that SFN dissolved in saline was a suitable vehicle; SFN in saline was able to induce Nrf2 and induce anti-inflammatory effects and therefore, saline was chosen due to its suitability for use as an *in vivo* vehicle⁴⁰. Other groups previously published *in vitro* studies had shown that key enzymes upregulated by SFN induced Nrf2 peaked around 12-48hr post administration and for this reason, a pre-treatment timing of 24hr was deemed appropriate to induce anti-inflammatory enzymes prior to the LPS challenge and coincide this with their peak upregulation. Additionally, effects of SFN appeared to be apparent in several other animal models, both peripherally and within the brain, at a starting dose of 5mg/kg. For this reason an initial protocol using i.p. injection with SFN pre-treatment at 5mg/kg dissolved in saline was used to investigate the effects of SFN in the model of cerebral inflammation was established in Chapter 4.^{25,41,42,241}.

Initial experiments conducted in this project using SFN dissolved in a saline vehicle did not appear to support the anti-inflammatory effects of SFN previously shown⁴⁰. The dose was increased to both 25 and 50mg/kg and the effects of leukocyte-endothelial interactions were visualised. Barring some alterations in the number of cells rolling through the microvasculature, there appeared to be little effect; and certainly no inhibitory effects of the visualised cellular inflammatory response within the brain (Figure 4.1). Potential reasons for these differences in the response to SFN seen here compared to other previously reported work may be due to differences in protocol for making up SFN, but it was apparent that in our protocol, SFN did not appear to altering leukocyte-endothelial interactions in the manner we had anticipated owing to the published anti-inflammatory effects within the literature⁴⁰.

Due to this disparity seen with respect to published work supporting anti-inflammatory effects associated with SFN and the lack of effect in the model used here, it was questioned whether SFN was stable in a saline vehicle. This led to the conduction of the liquid chromatography mass spectra to be performed on SFN that had been dissolved in saline, a corn oil and PBS mix or corn oil alone (Figure 4.2). Corn oil was selected as a vehicle due to its previously published used, and also the fact that it was less reactive with the isothiocyanate than saline or water²⁵. The outcome of these spectra suggested that SFN was indeed unstable in a saline vehicle, as demonstrated by the multiple small ionization peaks present (Figure 4.2a). However, when SFN was dissolved in either a corn oil and PBS

mix vehicle or corn oil alone much cleaner spectra were produced suggesting a cleaner product and therefore, much improved stability (Figure 4.2b and c). For this reason, experimentation with SFN in saline was no longer pursued and all further experiments were conducted with SFN in a corn oil and PBS vehicle.

Effects of SFN on leukocyte-endothelial interactions

Following on from these findings indicating the instability of SFN in saline, pre-treatment with corn oil and PBS was tested to ensure that this protocol would not interfere with the leukocyte-endothelial interactions we had previously seen with LPS (Figure 4.2/3). Data indicated that similar leukocyte-endothelial interactions occurred in the presence of this pre-treatment with a significant increase in the number of rolling and adherent cells when LPS treated animal were compared to their saline controls (Figure 4.3). Due to this finding it was concluded that pre-treatment with SFN did not alter the inflammatory cell interactions stimulated by LPS, and therefore suggested that corn oil/PBS was a suitable vehicle for *in vivo* SFN administration.

Pre-treatment with SFN at dose of 5 and 50mg/kg were then tested. It appeared that SFN at 5mg/kg was not able to alter leukocyte endothelial interactions. The number of rolling cells were comparable to those seen in vehicle treated animals, as was the rolling velocity and the number of leukocytes that were adherent to the vessel wall (Figure 4.4). However, when the dose of SFN was increased to 50mg/kg there did appear to be alterations in the leukocyte-endothelial interactions associated with LPS injection. Firstly, there was a marked elevation in the number of cells seen to be rolling through the microvasculature when compared to all other LPS treated groups (Figure 4.4a). These cells also appeared to be travelling at an increased rolling cell velocity when compared to animals treated with corn and LPS (Figure 4.4b). There was also a marked reduction in the number of adherent cells within the pial venules, meaning the number of adherent cells in SFN(50mg/kg)/LPS treated animals was no longer significantly different from those being treated with saline(Figure 4.4c). This suggested that SFN pre-treatment was capable of altering LPS induced leukocyte-endothelial interactions and may provide potential anti-inflammatory effects within the cerebral microvasculature. Taken together, these altered cellular interactions suggest that SFN (50mg/kg) pre-treatment is affecting the way leukocytes interact with the LPS stimulated cerebral endothelium, and could therefore, potentially produced anti-inflammatory effects.

These alterations seen in the experiments of Figure 4.4 could be suggestive of the ability of SFN to alter the expression of the adhesion molecules underlying the leukocyte-endothelial interactions induced via LPS injection. As mentioned earlier, these include adhesion molecules covering all phases

of the leukocyte adhesion cascade discussed at length by Ley *et al*¹. The expression of selectins regulates the primary interactions that occur between leukocytes and endothelial cells, and it has previously been shown that it is this adhesion molecule that mediates the increased number of rolling cells associated with LPS injection that were visualised in this project¹⁷⁰⁻¹⁷³. In order for leukocytes to progress on to develop firm adhesions to the vessel wall it is thought that both the endothelial cell and the leukocyte must both express fully functional and activated integrin molecules. Alteration of the ability of integrin molecules to undergo the conformational changes that support this firm adhesion would therefore subsequently effect interactions associated with the leukocyte adhesion cascade^{175,180}. The fact that this reduction in leukocyte adhesion seen in Figure 4.4c (following SFN (50mg/kg) treatment compared to corn oil/LPS treatment) is concomitant with an increased number of rolling cells (Figure 4.4a) suggest that there may have been a shift in these late stage adhesion molecules such as ICAM1 and/or β_2 integrins that support firm adhesion, but that the presence of earlier stage adhesion molecules involved in cell rolling such as the selectins and mucins remain potentially unaltered (see Table 1.1). Indeed, although not previously reported in neutrophils *per se*, the ability of SFN to alter the expression of cell adhesion molecules downstream of LPS challenge has been highlighted before. Both in endothelial cells and monocytes pre-treatment with SFN decreased the expression of late stage adhesion molecules following an inflammatory challenge^{38,242}. Interestingly, the level of ICAM1 expression specifically has not been shown to be altered via SFN treatment³⁸. Indeed, it may be that SFN is altering the β_2 integrin family that interact with ICAM1. This could be via interference with the intracellular signalling that mediates integrin activation following engagement of chemokines with their receptors; or via inhibiting the elongation, unravelling or stabilisation of the integrins that is needed to expose their high affinity β propeller head to engage ICAM1 and cause firm adhesion¹⁵²⁻¹⁵⁶.

Equally, SFN has been shown to alter the ability of TLR4 to respond to the presence of LPS through alteration of the MD2 accessory protein's LPS binding pocket^{243,244} which could potentially alter cellular interactions induced by LPS, however, this may be an unlikely explanation for the interactions seen here due to the remaining high levels of rolling cells and lack of SFN treatment to alter LPS induced cytokine production in blood samples taken and following the LPS challenge of human neutrophils (Figure 4.5 and 4.8).

Effects of LPS and SFN on peripheral cytokine production

In order to investigate the effects of SFN on cytokine production cytokine levels within the plasma were analysed. Since cytokines are released from a plethora of cell types, and are in turn capable of acting in an autocrine and paracrine manner, their presence within the plasma helps to give a snapshot

of the systemic effects of LPS and SFN on the underlying inflammatory response. A peripheral sample was deemed the most appropriate as it is known that LPS has systemic effects following *i.p.* injection as was used here, and therefore this sampling technique may better reflect any effects that SFN may have on alteration of the cytokine profile present^{82,130,245}. Furthermore, sampling by cardiac puncture allows a large, consistent sample to be taken and therefore allowed multiple cytokine analyses to be ran; thus generating a more rounded picture of any potential effects of SFN.

A cytometric BEAD array was used as this kit allowed sensitive quantification of a range of cytokines that are known to be involved in the inflammatory response to LPS such as TNF α , MCP-1 and IL-6. Due to the systemic sampling method the production of these cytokines could be owing to release from several cell types including neutrophils, monocytes, platelets and endothelial cells which are all thought to play a key role in the responses seen within this model^{228,246}.

TNF α and IL-6 are prototypic pro-inflammatory cytokines that have been widely shown to be rapidly released, in large amounts following the onset of inflammation in several pathologies^{247,248}. The rapid and marked increase in TNF α seen here is similar to that previously published in papers using similar protocols with peripheral sampling^{236,249}. Indeed, injection of TNF α alone can be seen to mirror many of the pathological effects that occur downstream of LPS injection highlighting the seminal role of TNF α in orchestrating the cellular response to LPS¹³⁰. Along with IL-1 β , TNF α and IL-6 act at their cognate cell surface receptors to induce intracellular signalling cascades that lead to the activation of various transcription factors (e.g. NF κ B, p38) leading to the upregulation of cell adhesion molecules (e.g. ICAM1) and production of other pro-inflammatory mediators such as ROS, COX-2 and iNOS (see Figure 1.1)^{130,250}. The ability of these aforementioned cytokines to stimulate neutrophil and endothelial cells; as well as playing a role in platelet activation, supports the findings here. Data generated in this project indicated that SFN pre-treatment did not affect the systemic levels of these two pivotal pro-inflammatory cytokines at this time point (TNF α and IL-6; Figure 4.6-7). Some *in vitro* work has previously shown that SFN treatment is capable of reducing TNF α mRNA, effects on actual protein levels appear to vary and therefore maybe dependent on time of sampling and the treatment protocol used^{40,251}. Interestingly, SFN has been shown to be able to induce IL-6 production *in vitro*, a finding that was abolished in the absence of Nrf2²⁵². Increased IL-6 in SFN treated groups was not seen here but whether this occurred at an earlier stage and may have potential priming actions could be of interest to investigate.

MCP-1 (CCL-2) was also found to be upregulated following injection with LPS (Figure 4.7). Contrary to earlier work suggesting a purely inflammatory role, it is now thought that MCP-1 plays an important role in controlling the inflammatory response associated with LPS. Removal of this chemokine leads to an increase in endotoxemia induced mortality²⁵³. It has also been suggested that MCP-1 may induce its protective effects via inhibition of IL-12 release from T cells and stimulating the release of IL-10²⁵³. Although very low levels of IL-12-p70 were seen in the samples taken here, there were seen to be considerable levels of IL-10 within plasma samples (Figures 4.9-10), specifically in LPS treated animals. The release of this cytokine in association with LPS highlights the multifaceted response of the immune system and TLR4 activation in response to LPS challenge. On top of this suggested immunomodulatory role of MCP-1, it has been suggested to play a role in the increased adhesion molecule expression and release of other pro-inflammatory cytokines such as TNF α ^{253,254}. This brings to light the dichotomous role for MCP-1, whilst supporting the findings presented here whereby high levels were seen in LPS treated animals that were seen to have increased leukocyte-endothelial interactions (Figures 4.4 and 4.6-7). It is of interest that MCP-1 acts in this dual modal manner to play potentially both pro-inflammatory and immunosuppressive roles since one of its primary actions has been suggested to be induction of monocyte chemo-attraction²⁵⁵. This monocytic recruitment may be involved in the initiation of wound healing and present an aspect of the resolution phase of the inflammatory response. Therefore, it is interesting that at this time point, MCP-1 is already elevated to this level. This could suggest a potential induction of the resolution phase, or an attempt of the immune system to regulate the inflammatory response, may have already begun in order to counter the pro-inflammatory effects of TNF α and IL-6 amongst other inflammatory mediators.

Equally, IL-6 has also been described to harbour these bimodal pro- and anti-inflammatory effects. Several papers have indicated the elevation of IL-6 following LPS challenge, often at a slight delay to TNF α and IL-1 β , likely indicating an inductive effect of one or both of these cytokine forerunners on release of IL-6²⁴⁸. The pro-resolution/anti-inflammatory effects of IL-6 may involve effects on leukocyte trafficking via alteration of chemokine expression, alongside subsequent effects of these cells once within the tissue^{256,257}.

Previously published data also suggests that production of IFN γ by T cells plays a corroborative role alongside IL-6 to reduce leukocyte trafficking²⁵⁶. This seems somewhat at odds with the trend towards a potential reduction in IFN γ induced by SFN here, when considered alongside the reduced leukocyte-endothelial interactions seen in the IVM studies. One explanation for this could be that SFN alters IFN γ at an earlier time point and this snapshot is simply showing the cytokine in a refractory phase. A more

detailed sampling protocol and examination of the cytokines across a boarder time frame would help to elucidate a clearer picture of the effects SFN may play on the temporal cytokine profiles.

This BEAD array kit also included analysis of IL-10, a cytokine whose release has received much interest in its role as a mediator of inflammatory resolution²⁵⁸. Interestingly, this cytokine was also upregulated in every LPS treatment group highlighting the complex nature of the cytokine response to an inflammatory challenge. Again, the data collected here does not suggest that SFN pre-treatment modifies the production of IL-10. Whether this is the case over the time period prior to sampling would require further investigation.

Although none of these cytokines are statistically altered following SFN treatment, they are all markedly increased in LPS treatment groups highlighting the potent and reactive systemic response of the host immune system to LPS challenge inflammatory nature of the host response to LPS. Whether the effects were mirrored in a more local cerebral environment or at the level of specific cell types (e.g. leukocyte subsets, endothelial cells, platelets) cannot be determined from this sampling technique but would potentially reveal specific drivers of this cytokine response.

Modulation of Nrf2 via SFN

Since it is the current dogma that the mainstay of SFN's effects are via modulation of the Nrf2-KEAP1 system, it was investigated whether at the time point used to visualise the cellular interactions administration of SFN was associated with altered nuclear Nrf2 expression. Cortical brain tissues were analysed since this corresponded to the tissue visualised during the IVM procedure. At this time point, there appeared to be no alteration in the nuclear expression of Nrf2 across any treatment group (Figure 4.8). Owing to the length of time that SFN had been administered to animals this finding is not necessarily unexpected. It is likely that SFN may have already induced Nrf2 translocation closer to the time of administration and its nuclear levels has subsequently decreased. Indeed, this finding is in agreement with others who have reported similar levels of total and nuclear Nrf2 levels following SFN pre-treatment at similar time points to those used here²⁵⁹. Previous groups have shown that SFN metabolites are detected within the brain over a rather acute time frame, as rapidly as ≈ 10 min and in other cases between 2-6hr with apparent clearance by 24hr^{24,40}. This suggests that peripheral administration with SFN rapidly reaches the brain and could, therefore represent the possibility for rapid alterations of Nrf2-KEAP1 interactions, leading to acutely increased levels of nuclear Nrf2 that would be missed at this time point.

Effect of SFN on peripheral leukocyte cell counts

The trends towards increased peripheral leukocytes counts seen here following LPS challenge is in agreement with previous data investigating the effects of LPS on peripheral blood cell counts. It has been shown that LPS induces both neutrophil and lymphocyte egression from the bone marrow, as well as causing a refractory drop in bone marrow leukocyte reserves²³⁵. Conversely, other groups have also reported a decrease in circulating leukocyte counts and attribute this to the sequestration in organ vascular beds²²⁸. These paradoxical findings may be explained by differences in sampling times, where cells may show an early increase in circulating numbers before dropping following recruitment and/or trapping within the microvascular beds of various organs. Why SFN would be associated with a significant increase in peripheral lymphocyte counts in saline treated animals compared with those being challenged with SFN is unclear. Whether these increased lymphocytes are involved in the altered cellular interactions seen with SFN is also not known. Previous work by the Kubes group has suggested that the percentage of cells interacting with the inflamed vasculature that are lymphocytes is remarkably low at four hours following LPS challenge, however, whether this is affected by SFN cannot be deduced from this work²²⁸. This could be further clarified by the use of specifically tagging different leukocyte subsets with different fluorescent markers.

Functional effects of SFN on human neutrophil cytokine production

The aim of the *in vitro* work using the human neutrophils was to investigate whether SFN could specifically alter neutrophil functional responses to LPS. Although the assay did show that LPS stimulated the release of IL-1 β when present in the cell culture medium, pre-exposure of SFN across a range of doses did not seem to affect this IL-1 β release (Figure 4.8). This indicated that SFN was not capable of affecting the functional response of neutrophils to LPS in this given protocol.

Possible reasons for this lack of effect may be due to an insufficient pre-exposure to SFN prior to treatment with LPS meaning an inability to upregulate sufficient anti-inflammatory mechanisms. Previously, it has been shown in other cell types that maximal phase II detoxification enzyme mRNA was upregulated at 12-24hr, with peak protein expression ranging from 24-48hr post-exposure to SFN²⁹. Therefore, if these enzymes need to be present and upregulated before the neutrophils are exposed to an inflammatory stimulus this may be a reason for the lack of effect of SFN seen in this study.

Conversely, other research has found that SFN afforded protection against LPS in other cell types when co-applied with an LPS challenge when measuring a range of inflammatory cytokine mRNA and transcription factor expression³⁰. Differences may be due to time at which the samples were taken or

potentially that particular cell population's exhibit different responses or time courses in their response to SFN or LPS.

This treatment protocol was chosen as it was found that with longer periods of *ex vivo* cell culturing leukocytes appeared to develop an apoptotic phenotype⁷¹. Therefore, in order to minimise activation caused purely by keeping the neutrophils *ex vivo* for a prolonged time frame, a treatment protocol of 4hr pre-exposure to SFN followed by 14hr LPS treatment was chosen. It was thought that this provided the optimal experimental design to pre expose the neutrophils to SFN and then induce an adequate inflammatory stimulus with LPS (as taken from previous work within the group).

Another potential reason for the lack of effect may be due to use of corn oil in the SFN vehicle, and the fact that this did not adequately mix within the cell culture medium. This may have led to inadequate exposure of the cells to SFN, and therefore, a lack of subsequent Nrf2 induction. This vehicle was used to avoid the necessity of exposing the cells to DMSO following the negative effect we saw when this was used in platelet experiments. Modification of this aspect of the protocol may shed further light of potential effects of SFN.

Summary

The work in this section highlights the ability of SFN to alter leukocyte-endothelial interactions following a systemic inflammatory response (initiated by LPS administration). LPS induced significant leukocyte-endothelial interactions which were concomitant with increased circulation neutrophils following exposure to LPS. Peripheral cytokine sampling highlighted the marked released of several pro-inflammatory cytokines associated with LPS which further supports the inflammatory response seen in the cortical microvasculature. A dose dependent effect of SFN treatment highlighted its ability to alter these leukocyte-endothelial interactions within the brain, with a potentially explanation being alteration in expression of adhesion molecules such as ICAM1 or the β_2 integrins; this suggestion would support the trend towards fewer adhesive cells following SFN (50mg/kg) and LPS treatment but a maintenance of elevated numbers of cells rolling through the microvasculature and no significant alteration in the number of cells circulating within the blood, with the exception of the lymphocyte difference seen between the SFN (50mg/kg) treated animals.

Further investigation into the underlying cell signalling events mediating these effects are needed to delineate how SFN is modulating these interactions. In particular, gaining a more detailed time course of Nrf2 translocation would aid understanding of the temporal dynamics of this transcription factor

induced by SFN and alongside this, a more detailed temporal cytokine profile would highlight the kinetics of cytokine production and the effect of SFN on these. The effect of SFN on cytokine receptors may also be of interest in defining how SFN alters cellular responses to inflammatory stimuli.

Additional experiments will also enable clarification of how SFN is mediating the shift seen in Figure 4.4 (adhesive to rolling phenotype). The use of monoclonal antibodies to ICAM1 or knockout mice would elucidate whether these effects of SFN on the number of adherent cells was ICAM1 dependent, and whether removing this adhesion molecule abolishes any effects of SFN. It would also be of interest to investigate whether this LPS response is associated with migration of leukocytes into the brain parenchyma and whether SFN alters this. A simple MPO assay could highlight the presence of reactive neutrophils across the treatment groups and highlight where this shift from cell adhesion to rolling was supported by decreased neutrophil reactivity within the brain. The use of specific fluorescently labelled antibodies to different leukocyte cell populations would also be of interest to delineate whether any of the alterations in cellular interactions following SFN treatment was driven by particular leukocyte subsets.

Chapter 5:

INVESTIGATION INTO THE EFFECT OF SFN ON PLATELET FUNCTION

5.1. Outline

Owing to the increasing knowledge of the role that platelets play during inflammation including:

- the ability to promote both homo- and heterotypic interaction of leukocytes and endothelial cells
- the fact that these cellular interactions support inflammatory function and migration of leukocytes and platelets into inflamed tissues
- the finding that the presence of both of these cell types within tissues both cell types can promote inflammation through the release of inflammatory mediators,

made investigation into the effects of SFN specifically on platelets an interesting and novel area of investigation.

Furthermore, at the outset of these experiments the specific effects of SFN on platelet function was not something that had been previously reported. Since it had been highlighted in the previous Chapter that SFN altered leukocyte-endothelial interactions stimulated in the LPS model, but that at the time points investigated there was no associated effect on cytokine production, Nrf2 expression or a functional effect on neutrophil using *in vitro* protocol, there was potential that SFN's effects may in part be explained by a platelet dependent function providing an exciting experimental question.

In support of conducting experiments to investigate the effect of SFN on platelet physiology in these experiments, it is the current dogma that the mainstay of SFN's effects involves the binding to key cysteine residues within its protein targets. It has previously been found that compounds capable of interacting with these groups are able to alter platelet physiology²⁶⁰. Hence, the aim of the work in this Chapter was to investigate what effect the presence of SFN would have on platelet responses.

Light transmission aggregometry was used to investigate how the presence of SFN altered platelet aggregatory responses to several physiological platelet agonists, and provide evidence as to whether SFN could specifically alter platelet function. Fura-2 loaded platelets were used to investigate whether these effects were associated with intraplatelet calcium movement. Lactate dehydrogenase (LDH) release was measured as a marker of cell membrane integrity and the potential of cytotoxicity associated with incubation with SFN.

5.2. Results

Investigation into dose related effects of SFN on isolated platelets

As a preliminary study, it was of interest to investigate whether SFN alone had any effect on platelet function. In order to do this, platelets were isolated, washed and resuspended in THB. The effects of the addition of SFN were then compared to that of a known classic platelet agonist, thrombin. Thrombin is a serine protease that acts to on both PAR1 and PAR4 to cleave the receptor leading to activation of platelet aggregation^{116,261}. This experiment allowed the aggregatory ability of the platelet isolates to be checked and subsequently visualise the effect of SFN to stimulate any aggregatory response. As Figure 5.1 highlights, addition of thrombin (0.1U/ml) was associated with a robust aggregatory response. A dose of 0.1U/ml Thrombin was chosen from previous work showing its consistent ability to induce a robust platelet aggregatory response across a sample of donors²⁶². Furthermore, addition of SFN over a range of doses so not associated with stimulation of platelet aggregation, suggesting that SFN does not act as a platelet aggregation agonist within these parameters.

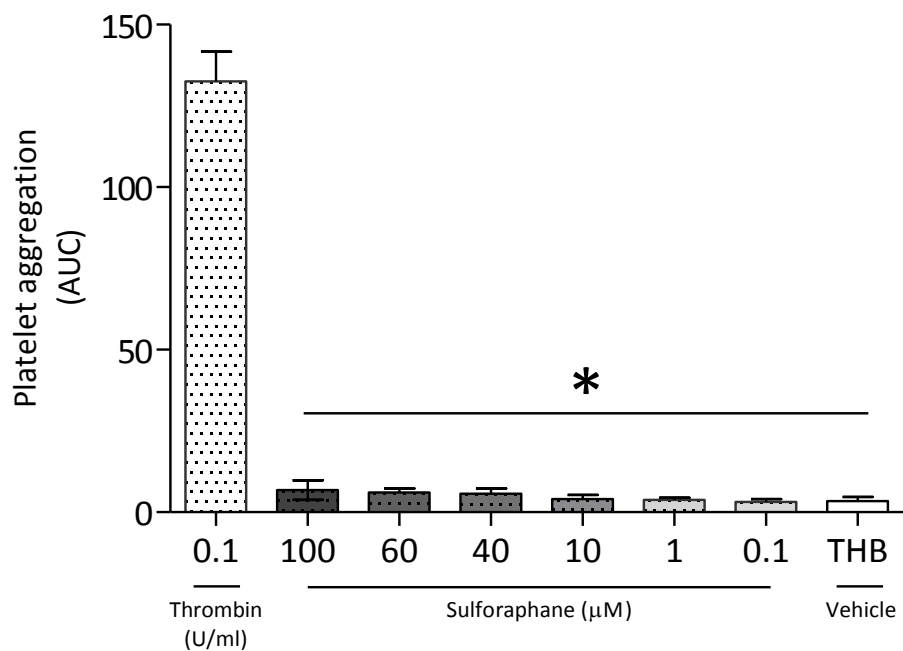


Figure 5.1: Treatment with a range of doses of SFN does not induce platelet aggregation.

Isolated platelets were washed and resuspended in THB at $250 \times 10^3/\mu\text{l}$. Platelets were added to cuvettes under constant stirring conditions (12000rpm) and the effects of SFN addition were compared to aggregation induced by thrombin (0.1U/ml) using area under the curve. All aggregations were run for 3min following addition of test compound. Data expressed as mean area under the curve $\pm$ SEM. * $P < 0.05$ compared with thrombin treated group using ANOVA with Dunnett's post hoc test. $n=3-5$ donors/group

Investigation into dose related effects of pre-incubations with SFN on isolated platelets to classic platelet agonists – thrombin, ADP and collagen

Next, investigation into whether the presence of SFN would either potentiate or inhibit the effect of subsequent agonist addition was undertaken. In these experiments platelets were incubated with SFN for either 2 or 30min followed by addition of a submaximal dose of agonist addition. Firstly, the effect of SFN pre-treatment on platelet responses to thrombin was investigated. Platelet aggregatory responses following a 2min incubation period with SFN prior to thrombin addition showed no effect, either potentiation or inhibition, on aggregatory responses to thrombin (Figure 5.2a). However, following a 30min incubation period with SFN, 100 μ M inhibited platelet aggregation (Figure 5.2b).

Two further platelet agonist (ADP and collagen) responses were also compared in the presence of SFN pre-incubation. Both ADP and collagen are weaker platelet aggregation agonists compared to thrombin, and therefore, may allow further effects of SFN to be seen. Collagen is present within the damaged vasculature as observed in atherosclerosis, and ADP is released by damaged cells and platelet dense granules^{105,137}. Using both these agonists, it was clear that there were trends present towards an inhibition of platelet aggregation (Figure 6.3-4). Using ADP in PRP, SFN pre-incubations at both 2 and 30min showed a dose dependent trend towards inhibition of platelet aggregation, with significance seen at 100 μ M SFN with 2min preincubation (Figure 5.3a).

Aggregations carried out with collagen highlighted the most potent inhibition of platelet aggregation following SFN pre-incubations. Both 2 and 30min incubations with SFN caused a dose dependent inhibition of platelet aggregation (Figure 5.4). Inhibition was most marked following 30min incubation periods with 40-100 μ M SFN significantly inhibiting aggregations. Significant inhibition was also seen at 60-100 μ M SFN following 2min incubation periods.

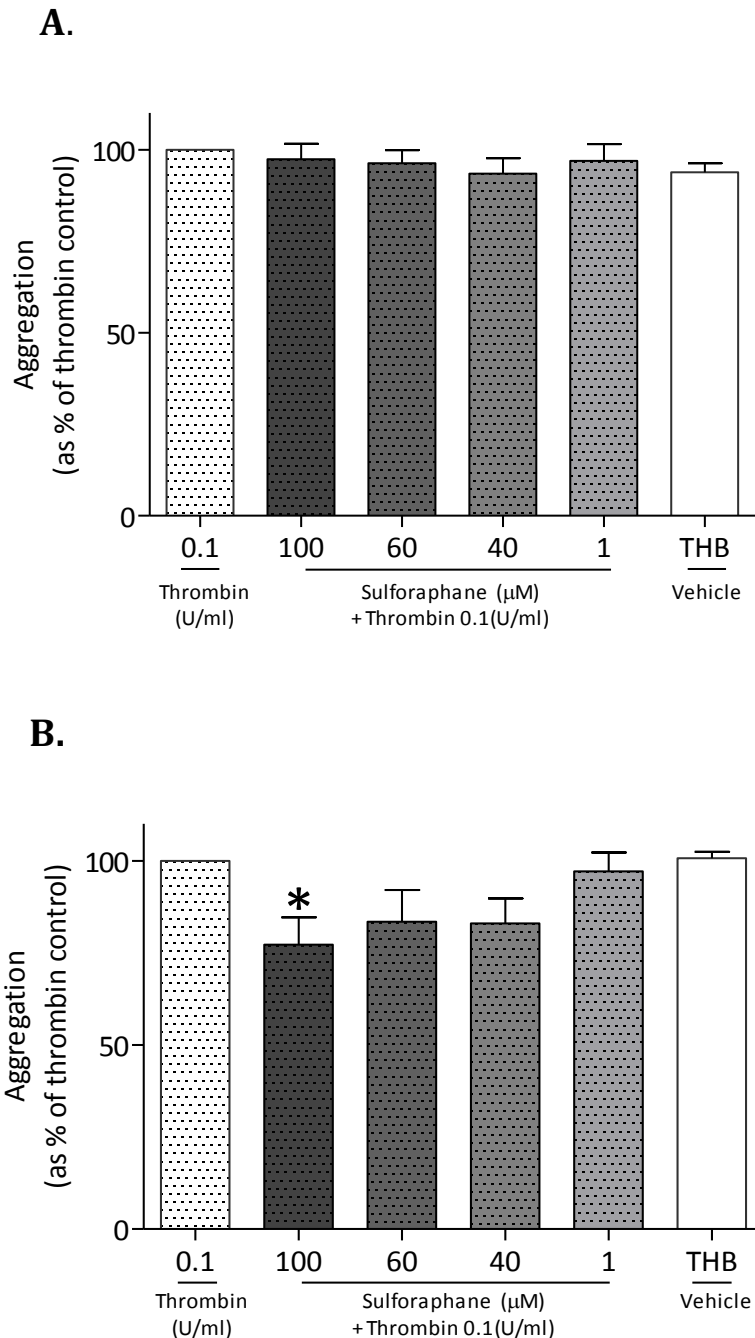


Figure 5.2: Pre-treatment with SFN induces dose and time dependent changes in the aggregation stimulated by thrombin. Isolated platelets were washed and resuspended in THB at $250 \times 10^3/\mu\text{l}$. Platelets were added to cuvettes under constant stirring conditions (12000rpm) and aggregation stimulated by thrombin (0.1U/ml) were compared to that stimulated by thrombin induced aggregations in platelets pre-incubated with SFN/THB (30min). A) 2min vs. B) 30min incubations. All aggregations were run for 3min following addition of test compound Data expressed as mean area under the curve $\pm$ SEM. * $P < 0.05$ compared with thrombin treated group using ANOVA with Dunnett's post hoc test. $n = 5$ donors/group

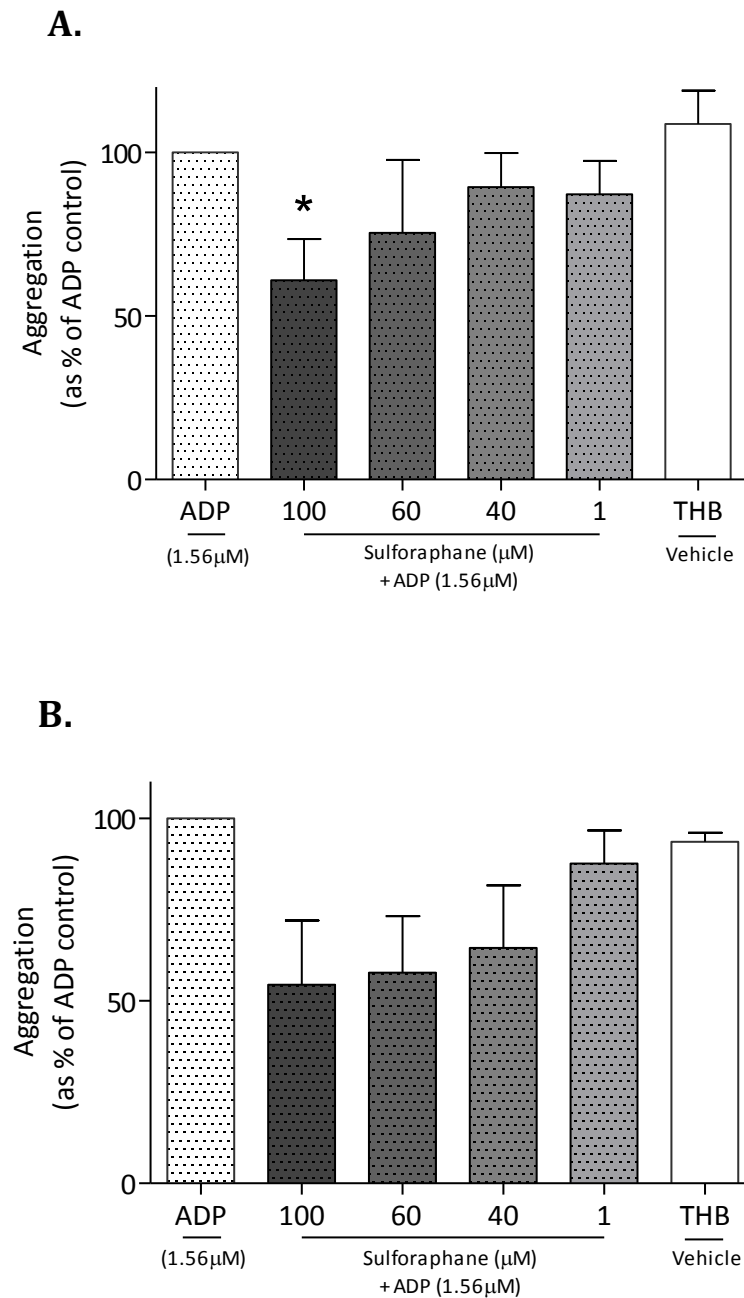


Figure 5.3: Pre-treatment with SFN induces dose and time dependent changes in the aggregation stimulated by ADP. PRP was isolated from whole blood. PRP was added to cuvettes under constant stirring conditions (12000rpm) and aggregation stimulated by ADP (1.56µM) were compared to that stimulated by ADP induced aggregations in platelets pre-incubated with SFN/THB (30min). A) 2min vs. B) 30min incubations. All aggregations were run for 3min following addition of test compound Data expressed as mean area under the curve $\pm$ SEM. * $P < 0.05$ compared with ADP treated group using ANOVA with Dunnett's post hoc test. n=3-5 donors/group

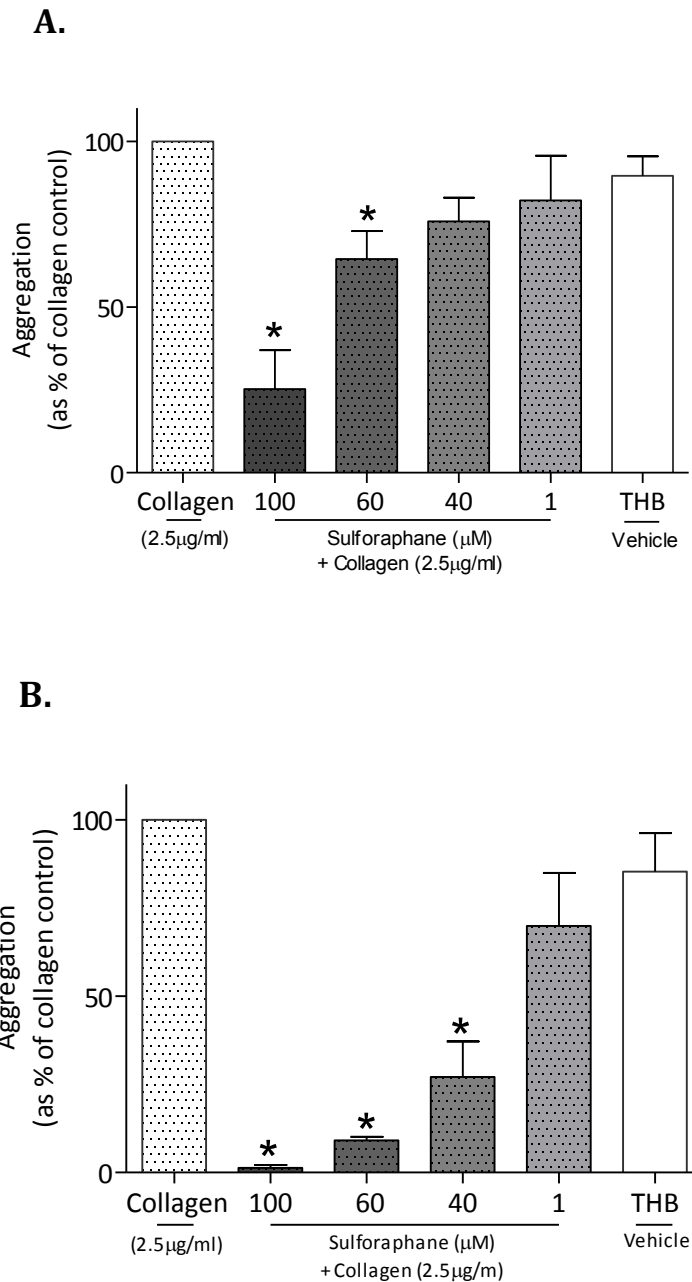


Figure 5.4: Pre-treatment with SFN induces dose and time dependent changes in the aggregation stimulated by collagen. Isolated platelets were washed and resuspended in THB at $250 \times 10^3/\mu\text{l}$. Platelets were added to cuvettes under constant stirring conditions (12000rpm) and aggregation stimulated by collagen (2.5µg/ml) were compared to that stimulated by collagen induced aggregations in platelets pre-incubated with SFN/THB (30min). A) 2min vs. B) 30min incubations. All aggregations were run for 3min following addition of test compound Data expressed as mean area under the curve $\pm$ SEM. * $P < 0.05$ compared with collagen treated group using ANOVA with Dunnett's post hoc test. $n = 3-5$ donors/group

Investigation into intraplatelet calcium movement following platelet exposure to SFN and associated LDH release

To investigate whether these anti-aggregatory effects are associated with any intracellular cell signalling events, platelets were loaded with Fura-AM fluorescent dye and fluorescence was measured (Figure 5.5). Thrombin (0.1U/ml) induced a rapid and marked increase in fluorescence, suggesting activation of intracellular cell signalling pathways and therefore, release of Ca^{2+} stores. However, neither addition of SFN or its vehicle THB, induced any alteration in fluorescence indicating that there was no release of intracellular Ca^{2+} stores indicative of a lack of intracellular signalling stimulation via SFN.

In order to examine whether SFN incubation is associated with any alterations in cell viability, a lactate dehydrogenase (LDH) assay was performed in order to measure any alterations in cell membrane viability (Figure 5.6). Following a 30min incubation period with SFN, LDH release was measured and compared to platelets that had been fully lysed in order to release their total LDH. Neither agonist addition; thrombin and ADP, nor a wide range of SFN doses were associated with LDH release when compared to complete cell lysis.

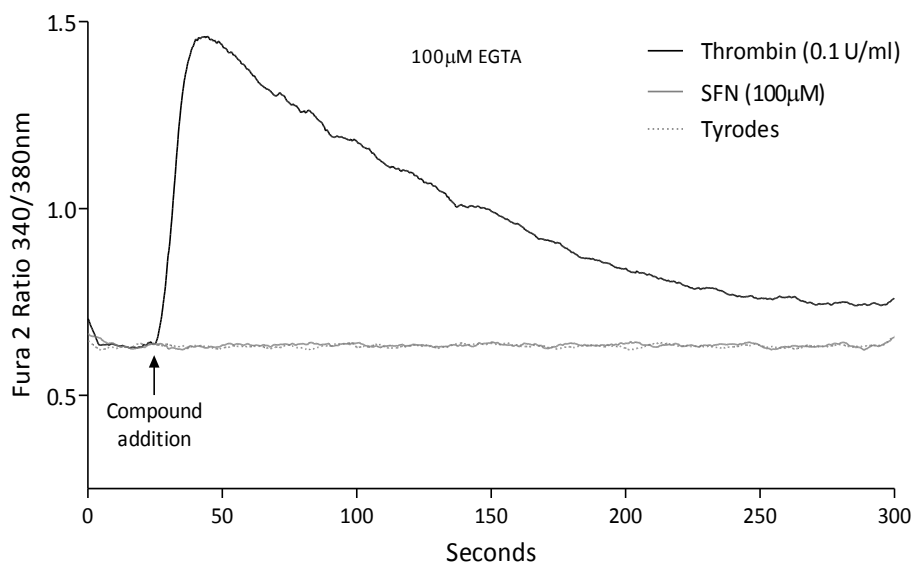


Figure 5.5: SFN treatment is not associated with alterations in resting intracellular Ca^{2+} release. Isolated platelets were washed and resuspended in THB at $250 \times 10^3/\mu\text{l}$, loaded with Fura2AM and allowed to equilibrate for 30min in the dark at 37°C . Platelet Ca^{2+} movement was measured as an alteration in light emission (340/380nm) in response to addition of classic agonist thrombin (0.1U/ml), SFN at (100μM) or vehicle treatment. Cuvettes were under constant stirring conditions (12000rpm). Graph shows representative trace from n=3 donors

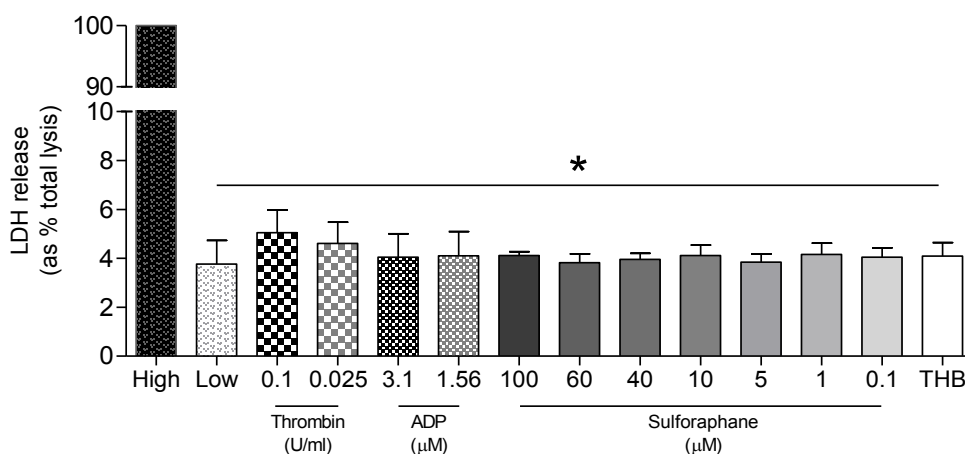
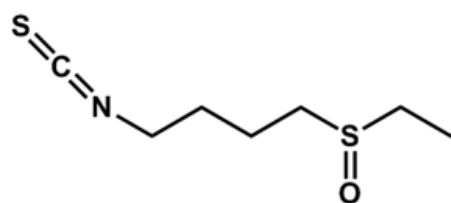


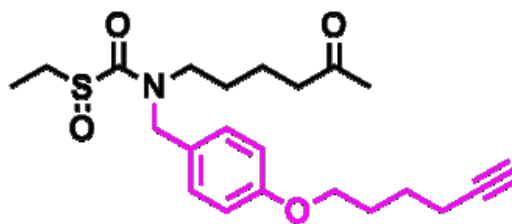
Figure 5.6: SFN treatment is not associated with alterations in LDH release following a 30min incubation period. Isolated platelets were washed and resuspended in THB at $50 \times 10^3/\mu\text{l}$, and incubated with SFN over various doses for 30min. Data expressed as a mean percentage of LDH released stimulated by complete cell lysis $\pm$ SEM. * $P < 0.05$ compared with collagen treated group using ANOVA with Dunnett's post hoc test. n=3 donors/group

Investigation into the effects of SFN like ABPs on isolated platelets and following pre-incubation periods prior to thrombin induced platelet aggregation

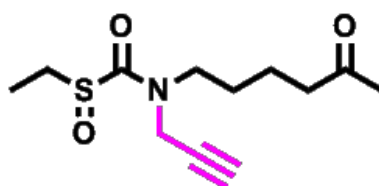
Following the inhibition of platelet aggregation seen following SFN pre-incubations above, we went on to conduct the aggregations with several activity based probes (ABP) (Figure 5.7). These compounds are SFN 'like' molecules that have undergone functional alterations to their isothiocyanate group. These modifications mean that unlike SFN, the ABP's have a higher binding affinity than SFN and therefore bind targets in a more permanent manner. The aim of using these molecules was to dissect the target molecules within the platelets that SFN was binding to. In order to test whether these compounds had similar effects to SFN on the ability to inhibit platelet aggregation, they were also run through the light transmission aggregometry protocol as described in Section 2.5. As was seen with SFN, addition of all ABP compounds and their vehicle to aliquoted platelet cuvettes did not stimulate platelet aggregation when monitored over a 3min period (Figure 5.8). Following a 30min incubation period at 100 μ M all of the compounds showed a trend to inhibition of platelet aggregation stimulated by thrombin (0.1U/ml) which was significant in both ABP1 and ABP2. When the dose of SFN was lowered to 10 μ M this inhibition of platelet aggregation to thrombin (0.1U/ml) was no longer present (Figure 5.9).



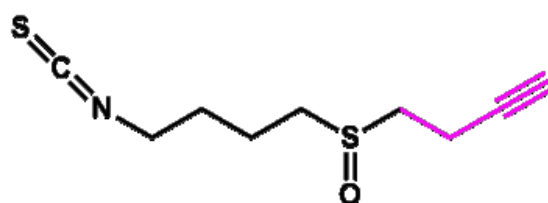
Sulforaphane



Sulforaphane ABP 1



Sulforaphane ABP 2



Sulforaphane ABP 3

Figure 5.7: Chemical structure of SFN and SFN like ABP compounds. SFN and SFN like ABP compounds showing various additional alkyne groups in pink to allow subsequent use of Click chemistry to isolate protein targets that the compounds have bound. (Clulow et al, *unpublished*)

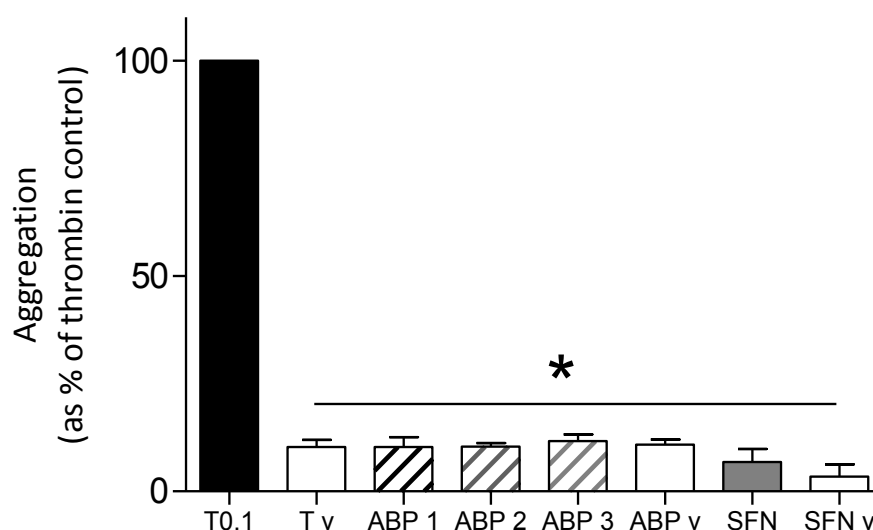


Figure 5.8: Treatment with a range of ABP compounds does not stimulate aggregation.

Isolated platelets were washed and resuspended in THB at $250 \times 10^3/\mu\text{l}$. Platelets were added to cuvettes under constant stirring conditions (12000rpm) and the effects of ABP (100 μM) compound addition were compared to aggregation induced by thrombin (0.1U/ml) using area under the curve. ABP vehicle contained (0.4% DMSO; final in cuvette). All aggregations were run for 3min following addition of test compound. Data expressed as aggregation as a percentage of that stimulated by thrombin control $\pm$ SEM. * $P < 0.05$ compared with thrombin treated group using ANOVA with Dunnett's post hoc test. $n = 3-5$ donors/group

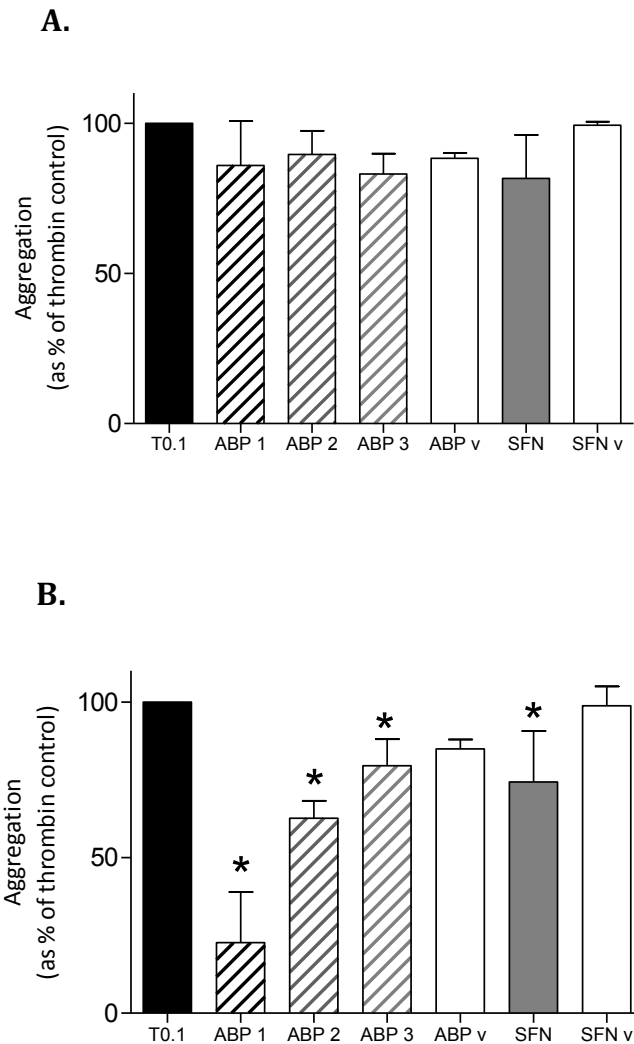


Figure 5.9: Treatment with a range of ABP compounds inhibits platelet aggregation induced by thrombin. Isolated platelets were washed and resuspended in THB at $250 \times 10^3/\mu\text{l}$. Platelets were incubated with ABP compounds A) $100\mu\text{M}$ or B) $10\mu\text{M}$ for 30min, then added to cuvettes under constant stirring conditions (12000rpm) and stimulated with thrombin (0.1U/ml). ABP vehicle contained (0.4% DMSO; final in cuvette). All aggregations were run for 3min following addition of test compound. Data expressed as aggregation as a percentage of that stimulated by thrombin control $\pm$ SEM. * $P < 0.05$ compared with thrombin treated group using ANOVA with Dunnett's post hoc test. A (n=2-3 donors/group) B (3-5 donors/group)

5.3. Discussion

The aim of the work in this chapter sought to investigate the effects of SFN on platelet function. Since the IVM work using the model of endotoxaemia presented in chapter 4 had highlighted the ability of SFN to alter leukocyte-endothelial interactions, there was a possibility that this could also involve modulation of platelet function. Previous work in several inflammatory models has shown a contributory role of platelets in the development and progression of inflammatory pathology, and the effect of SFN on platelet function was not something that had been specifically reported^{124,128}. Furthermore, it is known that platelet activation underlies the pathology associated with sepsis whereby patients are seen to have disseminated intravascular coagulation (DIC) and altered haemostatic factors²⁶³. Additionally, it is known that platelets express TLR4, and that presence of LPS can thereby alter their aggregatory and thrombotic platelet function^{246,249,264,265}. As a result, the work in this Chapter represented an exciting novel avenue of research into SFN's physiological effects.

Light transmission aggregometry provided an ideal starting point to study SFN's effects on isolated platelets. Use of this technique allowed investigation into whether the presence of SFN could directly affect platelet function in response to various aggregatory stimuli. Thrombin, collagen and ADP were used as platelet agonists; use of these three different agonists allowed investigation into the effects of SFN following platelet stimulation at distinct receptors (which in turn activate diverse platelet signalling pathways) to be explored. Furthermore, each of these agonists may be encountered physiologically in either stroke or sepsis making them relevant to this body of work.

Thrombin is a classic platelet agonist known to act via cleavage of the PAR1 and PAR4 receptors. Signalling through these receptors activates the platelets to undergo shape change and subsequently aggregate together. Under normal wound healing physiology, a local increase in the presence of thrombin activates platelets that are adhering to exposed collagen at the vessel wall. Release of platelet intracellular granules amplifies platelet recruitment, and thrombin is further involved in the stabilisation of the platelet plug via its role in fibrinolysis and consequent thrombus stabilisation. Aggregations to thrombin conducted here showed that a pre-incubation period of 30min, but not 2min, was associated with a modest inhibition of platelet aggregation. This suggested that SFN must in some way be altering the way in which platelets were able to respond to the presence of thrombin, either potentially at the receptor level, or via alteration of the intracellular downstream signalling cascades associated with PAR1 and/or PAR4. This will be discussed subsequently. These experiments were then conducted in response to addition of collagen and ADP.

Collagen is of particular pertinence to thrombotic risk since it is exposed following damage to the vascular wall as may be seen following injury or via rupture of an atherosclerotic plaque, and is non to be a risk for subsequent onset of ischaemic stroke^{266,267}. It activates platelet responses via the GPVI receptor and also via interactions with the integrin $\alpha_2\beta_1$. Collagen aggregations appeared to be most sensitive to exposure to SFN following both 2 and 30 min pre-incubation periods. Aggregation curves appeared to show that SFN delayed the onset and time taken for platelet shape change to occur and also slowed the onset of the overall aggregatory response. The working hypothesis is that this may be via SFN's ability to interact with and adduct to cysteine residues in protein key to the signalling cascade associated with collagen induced platelet aggregation. These ideas are expanded below.

Finally, ADP activates platelet responses via the purinergic receptors, P_2Y_{12} and P_2Y_1 . It is though that P_2Y_1 is predominantly involved in the initial shape change associated with platelet activation, whereas, P_2Y_{12} receptor underlies the subsequent onset of the aggregatory response²⁶⁸. ADP as a platelet agonist is of interest since it is known to be released via damaged cells and could therefore affect platelet activity within their vicinity, and furthermore, it is also released by activated platelets following exposure to other agonists such as thrombin and collagen. ADP induced aggregations showed a trend towards inhibition by SFN exposure following both 2 and 30min pre-incubations with 100 μ M SFN reaching statistical significance at 2min. Again, the presence of cysteines within the purinergic receptors may provide a rationale for these inhibitory effects, and is discussed below²⁶⁹.

After seeing these inhibitory effects associated with SFN pre-incubation to all of the agonists tested, lactate dehydrogenase release (LDH) in platelets incubated with a range of SFN concentrations was tested. LDH release as a measure of cell membrane integrity was used as it provided a rapid and sensitive indicator which could be reproducibly performed, and did not necessitate prolonged incubation periods with the platelets which may have itself affected results. The doses of SFN used in these platelet aggregations caused no greater LDH release than that associated with either vehicle treatment or platelets incubated alone. This suggested that the effects were unlikely to be due to disruption of cell membrane integrity causing a reduction in the platelets ability to respond to stimuli.

It was then of interest to investigate whether during this pre-incubation period with SFN there was an induction of platelet signalling pathways that were causing these protective effects. Using Fura-2 loaded platelets, it was apparent that addition of SFN to the platelet suspensions did not release intracellular Ca^{2+} stores following addition of SFN, and was therefore unlikely to be having its effects via activating signalling pathways. For this reason, the current hypothesis is that the effects observed

here are likely to be associated with alteration of platelet thiols that are involved in the initiation of platelet signalling cascades to various agonists.

This hypothesis is postulated based on the known evidence both of how SFN effects the interactions between KEAP1 and Nrf2, and previous thiol modulating agents have affected platelet function.

The current dogma is that SFN binds key cysteine residues within KEAP1, leading to a change in its conformation and subsequent release of Nrf2. KEAP1 is known to harbour an unusually high level of cysteines within its structure, highlighting its role as a cellular oxidative sensor and ability to be rapidly reduced¹³. SFN has been shown to bind many of these cysteines located throughout its structure²⁷⁰. It is still to be clarified how binding to different cysteines is physiologically relevant and may specifically alter interactions. In any case, the promiscuous binding of SFN to thiols therefore represents a plausible hypothesis for SFN's effects seen here. Furthermore, binding to these residues would also occur in the absence of cell signalling induction.

The role of surface thiols on platelets is also beginning to become better understood and of functional relevance. Vicinal thiols are free sulfhydryl groups which are found in close proximity to one another under normal physiological conditions. Their ability to interchange between dithiols and disulphide bonds appears to represent a form of functional control. Inhibiting these molecular conversions has been shown to affect platelet activation^{214,215}. Likewise, alterations in free thiols have been shown to be important to several integrins ($\alpha_1\beta_2$, $\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$) involved in platelet adhesion and that enzymes involved in this exchange are important for platelet function²⁷¹⁻²⁷³. This theory also supports the marked inhibition demonstrated in aggregations to collagen following incubation with SFN, since the main collagen receptor GPVI has been shown to contain a free -SH group in its cytoplasmic tail which is necessary for the homodimerisation of the two immunoglobulin domains in order to create a functional GPVI receptor²⁷⁴. If SFN was capable of binding to this thiol group, it may be able to inhibit successful GPVI dimerization and therefore inhibiting signal transduction at this receptor supporting the inhibition of collagen induced platelet aggregation seen here.

Therefore, if SFN were to be binding or interacting with some of these key cysteine containing proteins, it could support the inhibitory effects that are seen herein. It was for this reason that the work involving the ABP compounds was initiated. Firstly, similar aggregometry studies using pre-incubations were conducted to investigate whether the ABP compounds would have a similar effect on the platelets ability to aggregate. In all instances, results showed that the ABP compounds caused

an inhibition of the platelet aggregation. It is also clear that these inhibitions appeared to be different both between the ABP compounds and SFN, and also between the individual ABP compounds tested. This result was in part expected since the primary purpose of using these compounds was their ability to bind their protein targets in a permanent manner through their ability to form 'relatively stable' thiocarbamate bonds, whereas, SFN is known to bind in a reversible manner forming dithiocarbamate bonds which are kinetically labile²⁷⁵. The fact that there appeared to be marked differences between the ABP compounds' ability to inhibit the platelet aggregation to thrombin may be due to their differences in the strength of the bonds they form and proteins targeted. Indeed, taking into account previous work investigating ABP1, the data supports the finding that ABP1 causes the greatest inhibition since it has previously been shown to have the greatest potency in a cancer cell line, potentially indicating it as the compound with the greatest ability to bind its targets (Clulow, *unpublished work*).

Ongoing work with platelets and these compounds aims to further decipher the proteins targeted by these compounds to shed further light on the potential protein targets of SFN within platelets. Furthermore, previous studies looking into the effects of compounds that inhibit platelet activation through interactions with protein thiols have shown the reversibility of these effects through incubation periods followed by removal of these agents; this protocol reversed the inhibitory effects seen²⁷². Conducting experiments such as these may also shed further light on the potential of SFN having these effects through thiol interactions.

Inhibition of receptor function occurring downstream of SFN binding key extracellular cysteine has been indicated at other receptors in different cell types. TLR4 receptors isolated from monocytic cells exposed to SFN prior to LPS challenge had SFN adducts at specific extracellular receptor cysteine residues²⁴³. Further, effects of SFN extended to a decrease in key downstream signalling effectors, such as NF κ B and COX2²⁴³. Furthermore, this effect was reversed when either dithiothreitol or N-acetyl-L-cysteine, two thiol donors, were present indicating the reversible and thiol dependent nature of the inhibition²⁴³. This could support the effects seen here, especially since the most marked effects were seen in the collagen induced aggregations and the $\alpha_2\beta_1$ integrin involved in collagen activation of platelets has been seen to contain several free thiols which may provide the potential sites for SFN adduction. Indeed, it has been speculated that these thiol residues are necessary for the adhesion stimulated by the presence of collagen²⁷⁶. Molecular alterations in these sulfhydryl groups occur when platelets interact with collagen via the $\alpha_2\beta_1$ integrin, and therefore, it is possible that compounds that

effect the ability of this receptor to alter its binding affinity will therefore effect its ability to subsequently respond to the presence of collagen²⁷³.

The role of thiol interactions has also been indicated to be of significance in the P₂Y₁₂ receptor, indeed it has been highlighted as a potential mode of action associated with the inhibition of platelet activity associated with clopidogrel. These effects seem to be specific to the P₂Y₁₂ receptor and are dependent of the expression of two cysteine residues that form a disulphide bond²⁶⁹.

Other avenues of interest to explore with this work would be investigation into intracellular effects that SFN may be having on platelets. Several recent papers have highlighted the potential of SFN to alter thrombotic responses and several key intracellular signalling proteins using both animal models and in vitro experiments. As shown in work within this project Jayakumar *et al*, found that SFN pre-incubations were associated with a decrease in aggregatory reactivity of platelets in response to collagen, which also resulted in a decrease in Ca²⁺ release²⁰². Similarly, Oh *et al*, found responses in line with work conducted here in that SFN pre-incubations inhibited responses to collagen, ADP and thrombin aggregations, and that these inhibitory effects were most marked in collagen stimulated aggregations²⁰³. Likewise, Chuang *et al*, found dose dependent inhibitory effects following SFN pre-incubations following stimulation of platelet aggregation following stimulation at various receptors³⁶. However, some discrepancy has been shown in that Jayakumar *et al*, did not find inhibitory effects of SFN using thrombin as a platelet aggregation agonist; ADP induced aggregations were not investigated. This may be due to differences in vehicle or agonist doses used. Preliminary work for this project using DMSO as a vehicle appeared to mask the more subtle responses seen in ADP and thrombin induced responses, and other work in the group has previously found the solvent to be associated with cytotoxicity. Investigation into key cellular signalling pathways showed that the presence of SFN altered phosphorylation of PLC, PKC, MAP kinases and Akt, as well as decreasing OH[•] formation and increasing cAMP²⁰². Other effects of SFN downstream of inhibiting collagen induced aggregation include decreased thromboxane synthesis, reduced ROS production and decreased ATP release^{36,202}.

To investigate whether the *in vitro* effects presented here are relevant at a whole system level preliminary work using a light/dye model of thrombosis is being used in some preliminary experiments order to investigate the effects of SFN on thrombotic responses within the cerebral microcirculation. This work is on-going nevertheless, the early results are presented in appendix I.

5.4.Summary

Taken as a whole the work presented in this Chapter 5, alongside that recently published in the cited papers within, highlights the anti-aggregatory effects following pre-treatment with SFN. SFN did not directly stimulate any platelet aggregatory responses but was able to alter platelet responses to three key biological platelet agonists. The exact mode of action is unclear, but from the work presented it does not appear to be associated with an active cell signalling response. Additional studies are required to determine the exact molecular interactions by which SFN is inducing these changes. Work with the ABP compounds found that they too were able to alter platelet aggregatory responses. Further work with these ABP compounds will help to expose potential protein targets to which SFN may be binding within the platelets, with the hope being that this may shed light on the *in vitro* inhibition associated with SFN seen here. Equally, conducting some of the above aggregations in the presence of thiol donors such as N-acetylcysteine and dithiothreitol would be beneficial in order to elucidate if the anti-aggregatory effects seen here occur in a thiol dependent manner.

Chapter 6

CONCLUDING SUMMARY AND FUTURE WORK

6.1. Overall summary

As research into cerebrovascular diseases continues, the presence of inflammation underlying these pathologies appears increasingly apparent, both within the CNS and the peripheral circulation^{137,277}. Central to this ongoing inflammation is the presence of leukocyte-endothelial interactions via adhesion molecule expression, and release of bioactive molecules such as cytokines and ROS from the cells involved¹. At the outset of this project, the aim of this body of work was to investigate how SFN would alter cellular interactions induced by inflammation within the cerebral microvasculature.

In order to study these cellular interactions an appropriate model with which to induced leukocyte-endothelial interactions within the brain was required, along with a suitable technology with which to visualise these interactions. At the outset of this project the hope was that this model would be based around the cerebral inflammation induced by ischaemia-reperfusion injury downstream of middle cerebral artery occlusion. This would have provided an ideal pre-clinical model with which to investigate the ability of SFN to alter the leukocyte-endothelial interactions induced by IRI and as well as other pathological markers associated with ischaemic stroke. However, owing to the difficulties outlined in Chapter 3, the use of this model was not ethically just or scientifically satisfactory. Therefore, a second model was investigated. This model involved peripheral injection of LPS and was found to induce robust and reproducible leukocyte-endothelial interactions. One of the strengths of this model was that it provide reproducible data in order to study the leukocyte-endothelial interactions induced in the brain following an inflammatory stimulus as outlined in the initial aims.

As presented in Chapter 3, these interactions appeared to be time, but not dose, dependent. Therefore, to allow comparison to be made between results gained here, and a body of work published in the literature, it was decided that LPS injection at 0.5mg/kg at a 4hr time point represented the most useful experimental data with which to gain novel findings on the effects of SFN on inflammatory cell interactions and compare these with current knowledge of these inflammatory interactions within the brain.

Using this experimental protocol, it was found that a peripheral challenge with LPS caused an increase in the amount of leukocytes interacting with the blood brain barrier within cortical pial venules. Following treatment with LPS, leukocytes were seen to slowly roll along the venular endothelium with a concomitant increase in the number of cells that were becoming adherent. This leukocyte behaviour was specific to animals treated with LPS suggesting that peripheral stimulation of leukocytes via an

LPS challenge was sufficient to alter the way in which these cells acted within the microvasculature of the brain.

IVM provided a sensitive imaging modality with which to be able to observe these individual cellular interactions and subsequently allow offline analysis of the number of rolling cells, their velocity and the number of cells that were adherent to the vessel wall. Hence, this model provided the ability to study how SFN could alter the intravascular interactions of leukocytes and endothelial associated with inflammation. One downfall of this analysis was that it did not allow leukocytes to be followed extravascularly. The ability to do this with more power IVM technology could help to present a further parameter of the inflammatory response, and also, more information on whether SFN effects these later stages of inflammatory cell migration should any be seen.

In order to study any effects SFN may have it was necessary to find a suitable dosing regime and vehicle for the *i.p.* administration of SFN. Following a literature review it was deemed that an appropriate starting treatment protocol with SFN would involve 24hr pre-treatment with SFN prior to LPS, in order to elevate several phase II detoxification enzymes that had previously been associated with the anti-inflammatory effects of SFN prior to LPS challenge^{22,25,28}. Due to the fact that the effects to be studied were of an inflammatory nature, it was attractive to use saline as a vehicle for SFN as this had previously been used for SFN with positive anti-inflammatory effects shown and is widely considered not to affect normal physiological responses⁴⁰. However, in the early experiments that were conducted there appeared to be little indication of an anti-inflammatory effect associated with SFN (in saline) pre-treatment across a range of doses. As this appeared at odds with a body of published literature, mass spectrometry was used to investigate whether SFN was stable in a saline vehicle. Spectra produced indicated the presence of several breakdown products with SFN in a saline vehicle and therefore, spectra were repeated in corn oil and corn oil/PBS (10%) as alternative vehicles that had also been previously published^{25,259}. Spectra produced with SFN in either corn oil or SFN produced indicated much cleaner products indicating fewer breakdown products and greater stability. For this reason, corn oil/PBS was taken forward in order to provide greater stability but minimal corn oil administration due to the potential for it to interfere with potential inflammatory outcomes^{278,279}. It was reasonable to expect that the lack of effect seen with SFN dissolved in a saline vehicle may have been owing to its breakdown, and therefore, experiments were re-run using SFN dissolved in corn oil/PBS. A more in-depth understanding of the chemistry of SFN's isothiocyanate may have helped to avoid this downfall, but as previous groups had shown positive effects with SFN in a saline vehicle, the use of SFN in this vehicle was appealing.

Firstly, visualisation of the effect of pre-treatment with this new vehicle was tested to ensure that it did not affect the parameters being measured (as there are many groups investigating the effect of dietary oils and their constituent omega fatty acids on physiological responses^{278,280,281}). As leukocyte-endothelial interactions did not appear to be effected via the presence of corn oil, the ability of SFN pre-treatment in this vehicle was investigated. The current data suggests that SFN was able to alter the leukocyte-endothelial interactions associated with peripheral LPS exposure. Put in the most simplistic terms, when using IVM to investigate a potential anti-inflammatory agent, one may expect an agent to decrease all parameters of the leukocyte-endothelial interactions highlighted in Figure 1.3 - the thought being that decreasing these interactions would highlight a decreased inflammatory environment and therefore protect the visualised tissue from damage associated with the noxious inflammatory environment.

The hypothesis for Chapter 4 was that pre-treatment with SFN would induce Nrf2 regulated anti-inflammatory enzymes, protect the vasculature from inflammatory challenges thereby leading to a decreased in the leukocyte-endothelial interactions that were visualised. Indeed, the data presented in Chapter 4 suggests that SFN decreases the number of adherent cells; however, this was not associated with a concomitant decrease in the number of rolling cells. This is intriguing as it could suggest that SFN has altered the phenotype of the BBB endothelium, the leukocyte, an intermediary cell (such as platelet) or a combination of all of these to change the interactions that were visualised using IVM. One explanation could be that SFN has altered the ability of either leukocytes or endothelial cells to upregulate or increase the affinity of their adhesion molecules associated with firm adhesion, such as the β_2 integrins or ICAM1 respectively. Previous work has shown that SFN and other phytochemical pre-treatment of monocytes was able to alter their inflammatory adhesion molecule expression, and therefore adhesion; hence the potential for a similar effect *in vivo* on other leukocyte cell types may be possible^{242,282}. This would support the finding of decreased adhesion, and mean that these previously adherent cells could still present but only be supported by the weaker interaction associated with the selectins consequently, leading to the increase in the number of cells rolling through the pial vessels. In future work, it would be interesting to investigate how these interactions and the effects of SFN would be affected in the absence of the β_2 integrins or ICAM-1 to further elucidate whether these adhesions molecules were indeed a mechanism of action for the altered leukocyte-endothelial interactions seen here. This would help to elucidate whether this decrease in adhesion and shift towards increased numbers of rolling cells following SFN treatment was due to altered adhesion molecule physiology.

It would also be of interest to see whether this decrease in adhesion was teamed with a decrease in neutrophil presence within the brain parenchymal tissue. As investigation into leukocyte emigration was not possible here, the potential to use a simple MPO assay as a marker of the presence of activated neutrophils may be interesting, as well as the use of immunohistochemistry to detect and compare the presence of intracerebral leukocytes between treatment groups.

This effect seen on the leukocyte-endothelial interactions using IVM were not associated with alterations in cytokine profiles or Nrf2 nuclear expression taken from terminal samples. The data presented in Figure 4.5 failed to show any difference between cellular localisation of Nrf2 in any treatment, investigation into Nrf2 localisation at further time points would help to clarify whether alterations in this transcription factor varied over a broader temporal profile. This would help to determine whether the effects seen here were dependent or independent of Nrf2 transcription factor. This could also be supported by investigation into the expression of proteins of the glutathione system and HO-1 as a marker of phase II detoxification enzyme stimulation. In combination, these experiments may help to delineate a mechanism of action associated with the altered leukocyte-endothelial interactions presented here; as the cellular pathways underlying the effects presented in this thesis are not currently known.

Neither using blood samples from animals or LPS challenge of human neutrophils suggested that SFN was capable of functionally altering cytokine production downstream of SFN and LPS treatment. Again this could be due to the time point chosen and would benefit from further sampling at multiple time points. Also, the use of a vehicle with greater solubility would help to resolve potential issues faced with the *in vitro* corn oil treatment of human neutrophils. In the animal model however, it is possible that even though the cells in SFN treated animals seem to have shifted from an adherent to a rolling phenotype they still maintain the same functional capacity to produce cytokines.; *ex vivo* testing of isolated cells may shed further light on this.

To draw on the work presented in Chapter 5 highlighting the ability of SFN to alter platelet physiological responses, there is potential that SFN may alter leukocyte-platelet interactions thereby having a downstream effect on the ability of leukocytes to adhere to the inflamed BBB. Indeed, using several platelet agonists, pre-treatment with SFN decreased the ability of the platelets to respond to stimuli highlighting the ability of SFN to alter the platelets function. This work was of real interest since little work investigating the effect of SFN on platelet physiology had previously been published. The fact that SFN affected platelet responses could point to a possibility that SFN was capable of effecting

platelet-leukocyte interactions. To take this work forward, and tie it into the leukocyte-endothelial interactions it would be interesting to investigate further cellular interactions using FACS to study whether isolated platelet-leukocyte interactions were affected following exposure to SFN. *In vitro* work investigating the ability of SFN to affect platelet binding, for example to endothelial monolayers, could also aid to develop a broader picture of how SFN may alter various intercellular interactions. In doing these further studies, light may be shed on how SFN altered the platelet-leukocyte-endothelial interactions presented in Chapter 4; something that is currently lacking and would help to bring together the work in chapter 4 and 5. Further, along this vein of thought, it could be informative to conduct the LPS/SFN *in vivo* studies in mice lacking platelets. This would highlight both; differences in leukocyte response in the absence of platelets, and secondly, how SFN affects leukocytes downstream of LPS challenge may change in the absence of platelet – to highlight whether the platelet is a factor in the altered leukocyte-endothelial interactions following SFN treatment.

Work in Chapter 5 would also benefit from further investigation in to the specific mode of action by which SFN is affected platelet activation. To this end, I think it would be of interest to perform the studies using a similar protocol with the addition of a washing step or use of a thiol donor chemical such as DTT. This would highlight whether the effects of SFN were reversible (suggesting the potential presence of reversible underlying covalent bonds formed between the platelet and SFN), and also, whether there were via interactions with thiol groups as suggested in the discussion of Chapter 5. This would shed further light on the effects of SFN on platelet physiological and may also provide further mechanistic possibilities for Nrf2 independent cellular responses. Likewise, further work with the ABP compounds in both platelets and neutrophils would also help to elucidate cellular targets of SFN helping to clarify the mechanisms underlying SFN's alteration of platelet and leukocyte responses.

6.2. Concluding remarks

To this end, the work presented in this thesis has shown the ability of SFN to alter inflammatory leukocyte-endothelial interactions *in vivo* and platelet aggregatory responses *in vitro*. The aim to establish a suitable model with which to induce and visualise cellular interactions within the brain was met and this allowed the effects of SFN to be studied as outlined in the second aim. The cellular and molecular approaches used to study the underlying cellular changes were inconclusive in providing the mechanism of action by which SFN altered leukocyte-endothelial interactions and platelet aggregatory responses. For this reason the hypothesis outlined in the introduction cannot be fully accepted by the work presented here. Work did show for the first time that SFN treatment was associated with decreased cell adhesion following LPS challenge. In line with this was an unexpected associated increased numbers of rolling cells. Induction of Nrf2 or amelioration of the inflammatory profile was not found at the time points presented here. This poses the need for further research into the effect that SFN may have on platelet-leukocyte interactions to tie this work together. It also leaves open the mechanism of actions that underlie these leukocyte and platelet effects, whereby additional investigation is needed to shed light onto the pathways that SFN is interacting with to cause the end effects presented in chapter 4 and 5. The extra studies mentioned in this summary would help to validate the work shown in this thesis, and conclusively address whether the effects of SFN could indeed represent a therapeutically useful intervention for the treatment of inflammatory pathologies within the brain.

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Appendix 1

As much as *in vitro* aggregometry experiments provide a fantastic opportunity to investigate the effect of various platelet agonists and potential anti-aggregatory agents on isolated platelets, this system also has its down falls. Due to the inherent nature of the isolated platelets, the system creates a somewhat pro-aggregatory environment devoiding the platelet of physiological interactions which *in vivo* act to counter platelet stimulatory signals. For this reason it is important to take findings generated with light transmission aggregometry into more complex models to investigate whether compounds display similarly inhibitory effects in the presence of these various external influences and cell types. Several *in vivo* thrombosis models have aided further understanding of pathways involved in thrombus initiation and formation, and provide a useful tool to investigate potential therapeutic targets. The light-dye model used herein involves venous injection of a light sensitive dye, fluorescein isothiocyanate (FITC), in combination with epi-illumination via a xenon light source. This subjects a specific 100µm² area to photo-activation leading to subsequent platelet activation and thrombus formation. This thrombus formation can then be followed temporally in order to monitor development of vascular occlusion. Vascular occlusion occurs in both arterial and venous vessels depending on the vessel exposed to the xenon light source, although time of onset and occlusion has been shown to vary depending on the vascular bed used, underlying pathologies present and the vessels chosen^{249,283}. The light dye model produces thrombus formation in the absence of endothelial denudation; it is postulated that endothelial ROS production underlies the thrombus formation it induces²⁶⁵. Other models include the use of lasers or ligatures to physically damage the endothelium, injection of other photoactive dyes such as Rose Bengal and direct application of Ferric Chloride to exposed areas of the vasculature surface²⁸⁴.

Benefits of the use of the light dye model include; several vessels in one animal to be recorded due to the small area of the brain exposed to the light source at any one time allowing both arteriolar and venular thrombus formation to be visualised in one animal, minimal interference with the tissue is required reducing the likelihood of any tissue damage effecting the onset of platelet formation and the lack of complex surgery needed to expose specific vessels decreases surgical stress to the animal used.

As a side project to support the *in vitro* work presented in Chapter 5, thrombus formation in the cerebral microvessels used to investigate the effect of SFN on *in vivo* platelet responses to try and broaden the picture of SFN's effects on platelet cell function. Rather than solely being an initiator of pathology in cerebral infarction, vascular occlusion is being appreciated as event that occurs as part

of a multi-stage and –cellular process involving driven by underlying inflammatory processes²⁸⁵. Use of the light dye model allowed the study of intravascular thrombus formation following endothelial cell injury.

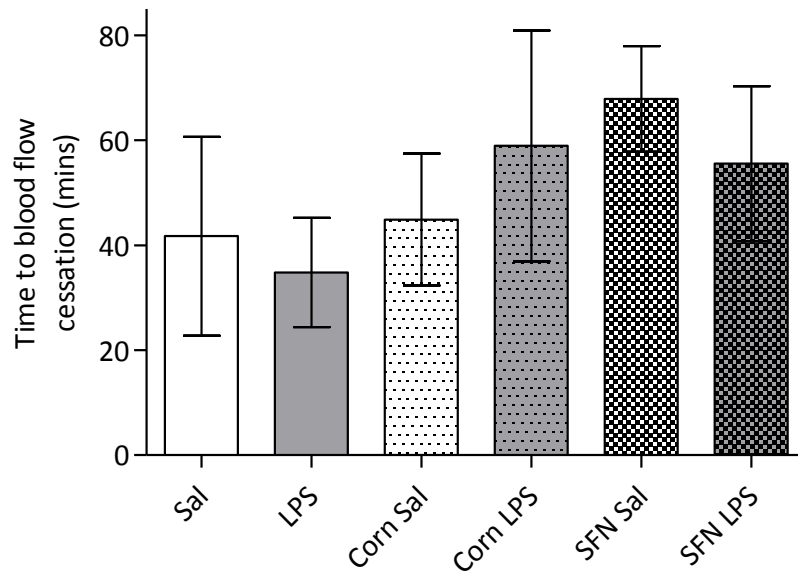
Results

Figure A.1 shows the findings from the light/dye thrombosis model. This was used to investigate whether an underlying inflammatory response would affect the onset of thrombosis in the cerebral microvasculature. Furthermore, it allowed visualisation of any effects afforded by pre-treatment with SFN. The data collected showed that complete occlusion of the cerebral microvasculature did not appear to be significantly altered when comparing mice that had received either saline or LPS *i.p.* injections. This was the case in both the arterial and venous side of the microvasculature that was analysed. However, when comparing the mean onset time of blood flow cessation, mice treated with LPS do appear to show an accelerated venule occlusion time when compared to saline treated mice ($34.81 \pm 10.40\text{min}$ vs. $41.75 \pm 18.75\text{min}$ to blood flow cessation respectively; Figure A.1b). This same trend was not seen in the arterioles of saline or LPS treated mice where values were much more comparable ($37.49 \pm 12.21\text{min}$ vs. $38.30 \pm 11.06\text{min}$ to blood flow cessation respectively; Figure A.1a).

Trends across the venules of mice treated with LPS, and receiving prior corn oil or SFN treatment were more difficult to deduce since corn oil alone seemed to delay the onset of blood flow cessation, albeit not statistically significantly, and this was not increased by SFN ($34.81 \pm 10.40\text{min}$ (LPS), $58.94 \pm 21.99\text{min}$ (Corn Oil/LPS) and 55.54 ± 14.78 (SFN/LPS); Figure A.1a). In saline treated animal, corn oil pre-treatment seemed to affect the blood flow cessation in venules to a lesser degree and therefore did highlight a trend towards an increase in the time to blood flow cessation by SFN ($41.75 \pm 18.95\text{min}$ (Sal), $44.90 \pm 21.99\text{min}$ (Corn Oil/Sal) and $67.89 \pm 10.05\text{min}$ to blood flow cessation (SFN/LPS) respectively; Figure A.1a).

Similar trends were noted when looking at the arterioles of mice receiving corn oil injections in that there appeared to be little effect of corn oil pre-treatment when mice received a saline injection ($37.49 \pm 12.21\text{min}$ (sal) vs. 36.43 ± 9.177 to blood flow cessation respectively (corn oil/sal); Figure A.1b), however, in mice receiving an LPS injection corn oil did seem to show some interaction in effecting the flow cessation of the arterioles ($38.30 \pm 11.06\text{min}$ (sal) vs. 47.96 ± 19.37 (corn oil/sal); Figure A.1b). SFN pre-treatment did not markedly alter arteriole occlusion time above any effects seen by corn oil whether followed by saline or LPS injection.

A.



B.

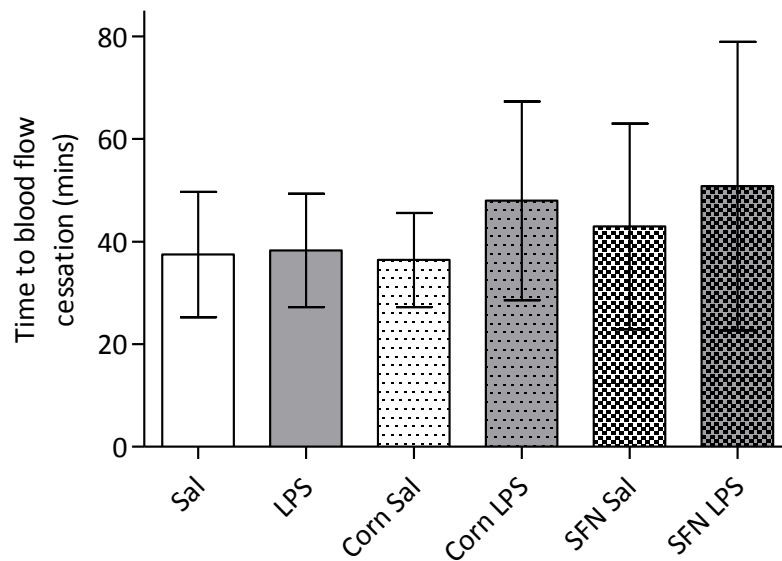


Figure A1: Cerebral vessel occlusion caused by light/dye induced thrombosis is unaltered following LPS administration or pre-treatment with SFN. Male, C57Bl6 mice were treated with SFN or vehicle corn oil (1hr, 5mg/kg, *i.p.*) prior to injection with LPS or vehicle saline (2hr, 1.5×10^6 EU/kg). Blood flow cessation time was measured A) cerebral venules and B) cerebral arterioles in straight and non-branching vessels with a diameter of 30-45 μ m. Bars represent the mean $\pm$ SD. n=3-6 mice/group

Discussion

This model involves injection of a FITC dextran, a photo-excitabile dye, into the venous circulation via a jugular cannula. Exposure of a $100\mu\text{M}^2$ area of the cortex to epi-illumination by a Xenon light source then leads photo-activation of FITC dextran and subsequent localised thrombus formation. This mode of thrombus formation is thought to be via production of superoxide molecules following activation of the FITC; this occurs in the absence of endothelial denudation²⁶⁴. Thrombi formed appear to be platelet rich, fibrin poor and display a general absence of leukocytes^{264,286}. Use of this model was advantageous as it allowed data to be gained on thrombus formation in arterioles and venules within one animal. It also allowed similar vessels to be visualised as were used within the endotoxemia study, and allowed temporal development of the thrombus formation to be visualised. Furthermore, the light/dye model has advantages over other models, such as topical FeCl_3 application, in that the former produces an intravascular insult leading to platelet activation, whereas, the later leads to outside-in damage of the vessel causing endothelial denudation and subsequent collagen exposure²⁸⁷.

The *in vivo* findings presented form part of an ongoing project and require further validation. At present, conclusions on whether pre-treatment of animals with SFN is capable of inhibiting subsequent thrombotic responses are difficult to draw.

In order to discuss the data as it stands, the arteriole findings across treatments groups will be compared, followed by effects within the venule treatment groups and then discussion about effects seen between these two groups. Potential factors causing these effects will then be postulated with relation to work previously published.

Time to blood flow cessation in cerebral arterioles was not affected by the presence of LPS. This is in opposition to previous work showing that exposure of mice to LPS leads to a reduction in the time to thrombus formation in several vascular beds²⁸³. It appears that pre-treatment with both corn oil and SFN appear to induce increased variability in the time to vessel occlusion, particularly in mice that are then subsequently exposed to LPS. Reasons for this are unclear since this variability was not seen when the corn oil vehicle was administered in the endotoxaemia model, however, the treatment protocol was different and may therefore cause different effects within the vasculature or at the vessel wall. This variability could be via an effect of the corn oil on the platelet, the endothelium or both. Taking this in account, the results are slightly ambiguous. There are slight trends towards a delay in onset to blood flow cessation with SFN, however, the variability seen makes conclusions uncertain. Larger 'n' numbers are needed to better power the study and hopefully remove some of the variability seen in

order to shed more light on potential SFN induced alterations in thrombosis. Indeed several groups' report 'n' numbers of up to 12 animals per group, suggesting these larger 'n' numbers are required for work with this model^{249,264}.

Lack of difference seen between saline and LPS treated animals may be associated with difference in biological activity of the LPS used. Several papers have reported alterations in findings between LPS lots; one paper reported having to give over three times the same dose of endotoxin in order to achieve similar thrombotic findings between subsequent doses of LPS²⁶⁴. Furthermore, different mouse strains have shown a difference in thrombotic responses associated with LPS, whether this can be extrapolated to potential differences in mouse colonies that would contribute to these effects is unknown²⁶⁴. It seems unlikely that this would be the case as these mice are routinely used for studies within this laboratory. Potentially, increasing the dose of endotoxin given in the experiments here would increase the difference in thrombosis induced vessel occlusion between saline and LPS treated animals to a level comparable to that reported elsewhere^{264,283}. Likewise, increasing the lamp power applied during vessel epi-illumination would be likely to increase thrombosis onset and may highlight greater differences between thrombosis kinetics in arterioles vs. venules.

Within the venules measured there also appears to be rather large variability in results seen supporting the need for further experimentation. However, taking the data as it stands there was a slight, although non-significant, reduction in blood flow cessation time following treatment with LPS versus saline alone. This is in agreement with previous findings which is thought to involve platelet derived TLR4 and not be associated with the increase in neutrophil adhesion associated with LPS treatment which we and others have seen within the cerebral microvasculature^{246,249,264,283}. Again, corn oil pre-treatment appears to alter thrombotic response, particularly in LPS treated animals, suggesting that this is potentially a real effect and not an artefact of small group sizes. Why this should occur in LPS treated animals remains unclear, but as mentioned above, with the level of variation seen in this group making conclusions seems somewhat futile. However, purely taking into account saline treated groups in the venules that were subjected to a thrombotic challenge, there does appear to be more of a discernible trend. Responses in both saline and corn oil pre-treatment followed by saline treatment are comparable, and furthermore mice receiving SFN pre-treatment followed by saline appear to show markedly delayed thrombus formation. This suggests that there is a possibility that SFN could modulate platelet position *in vivo*, and this is an effect that others have shown in several *in vivo* models. SFN pre-treatment was been found to decrease mortality associated with two different pulmonary thrombosis and also delay blood flow cessation in a light-dye thrombosis model with the

mesentery^{202,203}. Further support taken from ex vivo work conducted under flow conditions has also highlighted the ability of SFN to decrease thrombus formation³⁶.

Lack of difference between time to blood flow cessation in arterioles and venules is also in opposition to previous data, as well as the rather marked time to the thrombus vessel occlusion. Here, the lamp power that was used is considerably less than that previously published ($1.4\text{--}8\text{W}/\text{cm}^2$ vs. $1 \times 10^{-3}\text{W}/\text{cm}^2$) which would be the likely explanation for the slow thrombus formation seen here²⁸⁷. Indeed, thrombus formation using the light dye model has been shown to be a function of the light power and dye concentration and must therefore be closely monitored²⁴⁶. Therefore, it is highly probable that the lower light power used here explains this finding. Whether this decreased lamp power explains the lack of difference between arterial and venule thrombotic vessel occlusion is not certain. It may be that due to the lower power used, the increased time to thrombus formation causes the forming aggregates to be less stable due to the lower stimulus and therefore, more susceptible to being sloughed away. In fact, whilst recording the vessel occlusion it was often seen that occlusion occurred following accumulation of platelets downstream of the site of the light source which ultimately 'backed up' to clog the vessel. This may obscure the differences seen in arterioles vs. venules reported elsewhere. Furthermore, this slow onset time may be somewhat perpetuated by the need for prolonged use of anaesthesia, as anaesthesia has previously been shown to effect thrombosis formation²⁸⁶.

Summary

Validation of this cerebral thrombosis model will allow clarification of the effects of SFN on *in vivo* platelet activation. This model is a useful tool to study the effects of SFN in the whole organism with the multiple cell interactions that occur during platelet activation and thrombosis response. Increasing the power used with the lamp should help to be vessel occlusion times in line with those previously published and may aid differences between the arteriolar and venular sides of the cerebral circulation to become more apparent.

Appendix 2

Power calculations

The power calculations below were performed for the studies conducted in Chapter 4. They highlight the variability in the group sizes needed for the *in vivo* work conducted. Significance of graphs was only reported when the necessary number of animals was used as per the conducted power calculations. Power calculations for experimental data, especially *in vivo* work, are necessary to ensure that appropriate group sizes are used experimentally to warrant statistical significance is adequately reached. As can be seen below, the different parameters measured during the IVM experiments should have different levels of variability. This is in part an inherent problem with IVM, as lower numbers of rolling cells seen consequently give a smaller sample size with which to calculate a mean rolling velocity. For this reason, when interpreting the IVM data gained from the rolling cell velocity it is important to bear in mind the number of rolling cells present within the same treatment groups.

All power calculations were calculated using the formula:

Standardised difference (d) = Target difference/Standard deviation

$$2/d^2 \times C_{p, \text{power}}$$

The $C_{p, \text{power}}$ used was 7.9 (having 80% power to detect a difference with a probability of 0.05²⁸⁸).

Fig 4.3

A)

Sal vs LPS

$$2/(136.7/143.5)^2 \times 7.9 = 17.4$$

LPS vs Corn Oil/LPS

$$2/(20.8/50.8)^2 \times 7.9 = 94.3$$

B)

Sal vs LPS

$$2/(4.4/5.6)^2 \times 7.9 = 25.3$$

LPS vs Corn Oil/LPS

$$2/(5.9/0.9)^2 \times 7.9 = 0.4$$

C)

Sal vs LPS

$$2/(721.9/312.4)^2 \times 7.9 = 3.1$$

LPS vs Corn Oil/LPS

$$2/(16.9/150.8)^2 \times 7.9 = 1258$$

Figure 4.4

A)

Corn Oil/LPS vs SFN(50)/LPS

$$2/(149.7/71.2)^2 \times 7.9 = 0.003$$

SFN(50)/Sal vs SFN(50)/LPS

$$2/(259.4/71.2)^2 \times 7.9 = 1.2$$

B)

Corn Oil/LPS vs SFN(50)/LPS

$$2/(11.7/3.1)^2 \times 7.9 = 1.1$$

SFN(50)/Sal vs SFN(50)/LPS

$$2/(9.5/3.1)^2 \times 7.9 = 1.7$$

C)

Corn Oil/LPS vs SFN(50)/LPS

$$2/(376.5/155.3)^2 \times 7.9 = 2.7$$

SFN(50)/Sal vs SFN(50)/LPS

$$2/(697.2/155.3)^2 \times 7.9 = 0.8$$

Table 4.1

Neutrophils

Sal vs LPS

$$36.7 - 9.9 / 15.7 = 1.7$$

$$1.7 / 15.7 = 0.1$$

$$2 / (0.1)^2 \times 7.9 = 1336.6$$

LPS vs Corn Oil LPS

$$33.7 - 32.7 / 17.7$$

$$1 / 17.7 = 0.06$$

$$2 / (0.06)^2 \times 7.9 = 4950$$

Corn/Sal vs SFN/Sal

$$42.7 - 33.7 = 9$$

$$9 / 21.2 = 0.425$$

$$2 / (0.425)^2 \times 7.9 = 87.7$$

SFN/Sal vs SFN/LPS

$$42.7 - 35.7 = 7$$

$$7 / 27.7 = 0.253$$

$$2 / (0.253^2) = 247.412$$

Lymphocytes

Sal vs LPS

$$133.6 - 65.5 = 68.1$$

$$68.1 / 29.5 = 2.308$$

$$2 / (2.3)^2 \times 7.9 = 3$$

LPS vs Corn Oil LPS

$$241.7 - 61.7 = 179.4$$

$$179.4 / 9.3 = 19.3$$

$$2 / (19.3)^2 \times 7.9 = 0.042$$

Corn/Sal vs SFN/Sal

$$438.3-241.7 = 196.6$$

$$196.6/142.9 = 1.4$$

$$2/(1.4)^2 \times 7.9 = 8.347$$

SFN/Sal vs SFN/LPS

$$438.3-67.5 = 370.8$$

$$370.8/142.9 = 2.6$$

$$2/(2.6)^2 \times 7.9 = 2.3$$

Monocytes

Sal vs LPS

$$7.5-5.7/6.1$$

$$1.8/6.1 = 0.295$$

$$2/(0.295)^2 \times 7.9 = 181.557$$

LPS vs Corn Oil LPS

$$7.5-3.3/4.1$$

$$4.2/4.1 = 1.024$$

$$2/(1.024)^2 \times 7.9 = 15.068$$

Corn oil/LPS vs SFN/LPS

$$3.3-2.5 = 0.8$$

$$0.8/4.2 = 0.19$$

$$2/(0.19^2) = 437$$

Corn Sal vs SFN/Sal

$$8.3-3.3 = 5$$

$$5/4.1 = 1.220$$

$$2/(1.220^2) = 10.624$$

Platelets

Sal vs LPS

$$107900-93133 = 14767$$

$$14767/5446 = 2.712$$

$$2/(2.2712)^2 \times 7.9 = 2.149$$

LPS vs Corn Oil LPS

$$93133-89750 = 3383$$

$$3383/20349 =$$

$$2/(0.166)^2 \times 7.9 = 571.662$$

Corn oil/LPS vs SFN/LPS

$$98517-89750 = 8767$$

$$8767/30085 = 0.291$$

$$2/(0.291^2) \times 7.9 = 186.061$$

Corn Sal vs SFN/Sal

$$232233-100500 = 131733$$

$$131733/303128 = 0.435$$

$$2/(0.435^2) \times 7.9 = 83.66$$

