Systemic inflammation and its impact on the brain

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

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Stroke is a leading cause of death in the UK however there is only one current treatment, intravenous thrombolysis via administration of tissue plasminogen activator (tPA). The paucity of available treatments is not simply due to a lack of research as there have been many successful preclinical studies that have failed to translate to success in the clinic. The Stroke Therapy Academic Industry Roundtable (STAIR) have published several articles that outline a number of possible reasons for this lack of translation between preclinical and clinical research. One major reason highlighted is the failure to consider clinically relevant co-morbidities in preclinical studies.

Therefore the key objectives of this thesis were to: (1) determine whether central nervous system (CNS) changes occur in both animal models and patients with risk factors for stroke, (2) determine how neuroinflammatory changes induced in response to peripheral atherosclerosis are affected by the deletion of IL-1 signalling (3) establish whether a peripheral infection in atherosclerotic mice induces any cerebral ischaemic events and to determine the inflammatory response in both the periphery and the brain.

Neuroinflammation was assessed in patients at risk of stroke, the co-morbid JCR-LA rat and the atherosclerotic ApoE<sup>C/C</sup> mouse. PET imaging revealed microglial activation in the brain of JCR-LA (corpulent) rats and patients at risk of stroke. Microglial activation, vascular activation, leukocyte infiltration and focal lipid deposition were observed in the brains of atherosclerotic ApoE<sup>C/C</sup> mice. These findings show brain inflammation occurs in animals, and tentatively in humans, harbouring risk factors for stroke.

Neuroinflammation was assessed in ApoE<sup>−/−</sup> mice crossed with IL-1 type 1 receptor deficient mice (ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup>) and both neuroinflammation and systemic atherosclerosis was assessed in ApoE<sup>−/−</sup> mice treated with an anti-IL-1β antibody. ApoE<sup>−/−</sup> mice fed Paigen or Western diet develop vascular inflammation, microglial activation and leukocyte recruitment in the brain, which are absent in ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup>. Systemic neutralisation of IL-1β with an anti-IL-1β antibody reversed aortic plaque formation and reduced inflammatory cytokine expression in peripheral organs. In the brain, vascular inflammation and leukocyte infiltration into the choroid plexus were reversed by IL-1β blockade in animals fed a Paigen diet. These results indicate that IL-1 is a key driver of systemically-mediated cerebrovascular inflammation and that interventions against IL-1β could be therapeutically useful in atherosclerosis, dementia or stroke.

ApoE<sup>−/−</sup> and C57BL/6 mice infected with Streptococcus (S.) pneumoniae were assessed for spontaneous stroke events, neuroinflammation and systemic inflammatory responses to infection. Infection with S. pneumoniae in atherosclerotic mice did not induce spontaneous stroke. Raised levels of vascular activation were observed in all mice and an alteration in leukocyte accumulation in infected atherosclerotic ApoE<sup>−/−</sup> mice. T cell, B cell and granulocyte responses to both diet and infection were found to differ between ApoE<sup>−/−</sup>
mice and control, C57BLJ/6, mice. Levels of the proinflammatory cytokines IL-1, IL-6 and IL-17 were also increased in response to 
\textit{S. pneumoniae} infection in plasma, spleen and liver. These data indicate that atherosclerosis and 
\textit{S. pneumoniae} infections not only have systemic inflammatory mechanisms but also effects that extend to the brain.

Overall these findings demonstrate that risk factors for stroke cause alterations in inflammation in the brain. Therefore modelling of these risk factors is essential in future preclinical stroke research if new treatments for stroke are to be identified.

**Declaration**

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Caroline V Drake

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# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>non-infected ApoE&lt;sup&gt;−/−&lt;/sup&gt; mice fed a chow diet</td>
</tr>
<tr>
<td>ACP</td>
<td>activated protein C</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-l-5-methyl-4-isoxazole-propionate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>non-infected ApoE&lt;sup&gt;−/−&lt;/sup&gt; mice fed a Paigen diet</td>
</tr>
<tr>
<td>Apo E&lt;sup&gt;−/−&lt;/sup&gt;/IL-1R1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Apo E&lt;sup&gt;−/−&lt;/sup&gt; interleukin-1 receptor&lt;sup&gt;−/−&lt;/sup&gt; animals</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>ApoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Apolipoprotein E&lt;sup&gt;−/−&lt;/sup&gt; knockout mouse</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td><em>Chlamydia</em></td>
</tr>
<tr>
<td>CBA</td>
<td>cytometric bead array</td>
</tr>
<tr>
<td>CC</td>
<td>non-infected C57BL/6 mice fed a chow diet</td>
</tr>
<tr>
<td>CCL5</td>
<td>chemokine ligand 5 (RANTES)</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CP</td>
<td>non-infected C57BL/6 mice fed a Paigen</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CXCL</td>
<td>chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidene</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>distilled water</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte-stimulating colony factor</td>
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<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
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<tr>
<td>IAC</td>
<td>infected ApoE&lt;sup&gt;−/−&lt;/sup&gt; mice fed a chow diet</td>
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<tr>
<td>IAP</td>
<td>infected ApoE&lt;sup&gt;−/−&lt;/sup&gt; mice fed a Paigen diet</td>
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<tr>
<td>IBA1</td>
<td>ionized calcium binding adaptor molecule-1</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>ICC</td>
<td>infected C57BL/6 mice fed a chow diet</td>
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</table>
ICP infected C57BL/6 mice fed a Paigen diet

IFN interferon

IL interleukin

IL-1 interleukin-1

IL-10 interleukin-10

IL-17α interleukin-17 alpha

IL-1R1 interleukin-1 receptor-1

IL-1Ra interleukin-1 receptor antagonist

IL-1α interleukin-1α

IL-1β interleukin-1β

INF-γ interferon-gamma

KC CXCL1

l litres

LDL low density lipoprotein

LPS Lipopolysaccharide

MCAo middle cerebral artery occlusion

MCP-1/CCL2 monocyte chemoattractant protein-1

mg milligrams

ml millilitres

MMP matrix metalloproteinase

n.d. not detectable

N₂O nitrous oxide

NaN₃ sodium azide

NMDA N-methyl-D-aspartate

O₂ oxygen

P. Porphyromonas

PBS phosphate buffered saline

PET positron emission tomography

PFA paraformaldehyde

S. Streptococcus

SCID severe combined immunodeficiency

SD standard deviation

SSC side scatter

STAIR Stroke Therapy Academic Industry Roundtable

STWS Scotts tap water solution

T₉ T cytotoxic

T₉ T helper

TNF tumour necrosis factor

tPA tissue plasminogen activator

UK United Kingdom

VCAM-1 vascular adhesion molecule-1

VLDL very low density lipoprotein
Alternative format for submission

This thesis has been submitted in the alternative format to allow the incorporation of published and submitted papers. The format consists of a general introduction; three stand alone chapters each presenting a paper, either published or submitted for publication; and a general discussion followed by references. This format has been approved by the Faculty of Life Sciences for submission (presentation of theses policy, section 7).
Acknowledgements

I would firstly like to thank my supervisors; Stuart Allan, Adam Denes and Nancy Rothwell and my advisor Jaleel Miyan. No matter how busy Stuart was he always found time for me and showed me the correct way to tackle a conference (talks all day, pub all night). He always talks about how much I have grown during my PhD and although he would say the change was down to me, I know a big part of that was due to his tremendous guidance, and for that I am truly grateful. Adam, who was charged with the task of being my day to day supervisor, quickly taught me the joys of immuno. His endless patience, abstract sense of humour, enthusiasm for new data (even when not significant) and brilliant mind are inspirational.

The lab quickly became my extended family and was not only full of fantastic scientists but also a really fun place to work. I’d like to thank Deb and Angela for helping to sort out things that I had left to last minute (often) and always having the door open for a chat, whether that be about science or a new dress. Cath, who not only provided great scientific discussions but was also there to witness the events of the Berlin conference. Barry for proving that hand perfusion is a valid method and to Dave, whose dry banter never failed to entertain. I must also thank Hannah, Emily, Jesus, James, Sarah G, Herve, Holly and the rest of the lab who shared in the ups and downs of science and made everyday a pleasure.

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Most importantly, I’d like to thank my family; my brother, mum and dad, who have always stood by any decision I have made and shown a continued interest (sometimes blank eyed) in the research I was doing. I am truly blessed to have the support that they have given me and appreciate everything they have ever done for me.

Finally I would like to thank Simon Luckman, the IMB and BBSRC for finding me to be a worthy PhD candidate and funding me during my 3 years.

So it has been 3 years full of science, friends and some amazing times but all good things must come to an end; therefore sadly, so does this.
Chapter 1

Introduction
1.1 Overview

Stroke is defined as a reduction in blood supply to the brain, resulting in lack of oxygen and energy failure, neurological dysfunction or death (Baron, 2001). It is a leading cause of death and long term disability in the UK., with an estimated 110,000 people each year suffering first strokes (Scarborough et al., 2009). Due to the large number of people affected it is estimated to cost the NHS £8 billion per year, mainly for aftercare rehabilitation (Scarborough et al., 2009).

Despite the high morbidity and mortality rates, the only current treatment for ischaemic stroke is intravenous thrombolysis via the administration of tissue plasminogen activator (tPA) (Wayner et al., 1995). The lack of treatments is not simply due to a deficit in research as there have been many failed clinical trials, which appeared promising pre-clinically, and numerous pre-clinical studies attempting to find new therapies. The Stroke Therapy Academic Industry Roundtable (STAIR) discussion outlined a number of reasons for these failures and a major finding was a lack of translation between pre-clinical and clinical research (Fisher et al., 2009). An example of this lack of translation is poor modelling of risk factors for stroke.

The major risk factors for stroke include hypertension, age, atherosclerosis, infection, obesity and diabetes. Although these risk factors have been shown to have an inflammatory component the effect this has on the brain prior to stroke has not previously been studied. Therefore, this thesis will examine how the stroke risk factors atherosclerosis and infection affect neuroinflammatory responses in the brain and whether any effects are dependent on the pro-inflammatory cytokine interleukin-1 (IL-1).

The introduction will firstly outline ischaemic stroke, current treatments and possible reasons for the lack of success in finding a widely applicable treatment. Secondly, the inflammatory response will be outlined, followed by the contribution of inflammation to stroke with a focus on the proinflammatory cytokine IL-1. Finally the risk factors for stroke will be discussed, focussing on their contribution to increased stroke risk and the underlying contribution of inflammation.
1.2 Stroke

Stroke can be classified as either haemorrhagic (20%), which is caused by the bursting of a blood vessel in the brain (Warlow et al., 2003), or ischaemic (80%). Ischaemic stroke involves the occlusion of an artery supplying blood to the brain, with over 65% of cases arising due to blockage of the middle cerebral artery (Hossmann, 2008). In this thesis the terminology stroke will refer to only ischaemic stroke and haemorrhagic stroke will not be discussed.

1.2.1 Treatments

The outcome following an ischaemic injury depends on several factors including age, gender, occlusion duration and location, treatment and the level of reperfusion. Outcome ranges from death, through varying stages of neurological deficit to complete recovery. Despite the high morbidity and mortality rates the only current treatment for ischaemic stroke is intravenous thrombolysis via the administration of tPA (Wayner et al., 1995). The mechanism of action of tPA involves the breakdown of the occluding thrombus, allowing restoration of blood flow and reperfusion of ischaemic tissue, resulting in a reduction in the infarct volume and thus an increased survival of functional neurones (Grotta and Alexandrov, 1998). With an increasing number of surviving neurones, patients had a significantly improved neurological recovery and showed fewer symptoms such as one-sided weakness (Hacke et al., 1995). However, tPA is only applicable in confirmed ischaemic stroke and must be used within 4.5 hours of vessel occlusion, to avoid increased risk of a subsequent haemorrhage (Khaja and Grotta, 2007).

1.2.2 Failures in current research

Although only one treatment for stroke exists, many preclinical and clinical studies have been performed in the search for further treatments. These include those focused on further reperfusion therapies and neuroprotective agents to reduce neuroinflammation associated with stroke and rescue of dying cells (Cohen et al., 2011; Noorian et al., 2011). The failure of these treatments in a clinical setting caused stroke researchers to search for reasons why promising preclinical studies had failed. In 2009 the most recent STAIR discussion found a number of issues with the way current research is performed and how relevant it was to those patients suffering strokes (Fisher et al., 2009). The main observations were that current research had a lack of translational relevance to the clinic (Figure 1).
Figure 1. Mismatch between experimental animal modelling and stroke patients. The STAIR discussion highlighted a number of current problems with stroke research and a mismatch between experimental studies and stroke patients was found to be an issue which could be contributing to a lack of new therapies. The main mismatches identified are outlined above.

Most studies performed were found to use mainly young, healthy animals, despite the fact that clinically stroke affects mainly the elderly population. The damage occurring after experimentally induced stroke differs between young and aged animals and thus treatments used in young animals may not be relevant to the elderly population (Sieber et al., 2011). The population of animals used in preclinical studies is typically very homogenous, usually being male animals from one particular strain, which is often inbred. Genetic variability is therefore limited, and there is a lack of consideration of the effect of gender, despite the fact stroke affects both men and post menopausal women equally (Seshadri et al., 2004; Lisabeth et al., 2009). In preclinical studies, the approaches used to induce stroke typically cause reproducible injury that is located to one region (Atochin et al., 2004), but in the clinical situation strokes can occur in any region of the brain and the size of damage varies enormously between cases (Carmichael, 2005). Although most recent studies determine the effect of agents delivered post-stroke, much of the early work on neuroprotectants gave the treatment pre-stroke or immediately after occlusion. This does not match the clinical situation and there is a real need therefore to look at delayed administration that fits with the time-window of the patients presenting to the hospital. A further finding of the roundtable discussion was that studies are performed using healthy animals. Stroke patients rarely present without pre-existing risk factors for stroke and are usually found to be co-morbid with multiple risk factors such as atherosclerosis, obesity, diabetes and hypertension. Animal models with these risk factors have been shown to have a worse outcome following stroke.
(Terao et al., 2008). Most diseases, which are risk factors in stroke, are found to have a high level of associated systemic inflammation, such as raised levels of inflammatory molecules for example Interleukin-6 (IL-6) and C-Reactive Protein (CRP), which not only increases the risk of stroke but also can affect post-stroke outcome (Dandona et al., 2004; Hansson and Libby, 2006; Savoia and Schiffrin, 2006). Little is currently known about how the pre-existing inflammation present in both co-morbid patients and animals affects the inflammatory status of the brain, and therefore this will be the focus for some of this project. Stroke itself is known to have a large inflammatory component and therefore further understanding of these inflammatory mechanisms could provide avenues for new therapies to be developed.

1.2.3 Mechanisms of injury

The damage caused by occlusion can be divided into two distinct regions, the core and the penumbra. The core region is adjacent to the focus of ischaemia and the hypoxia experienced by neurones in this region means that the metabolic activity required to maintain structural integrity has been lost (Hossmann, 2006). Therefore this brain tissue, consisting of mainly necrotic tissue, is considered unsalvageable. Surrounding the core is the penumbra, this region contains compromised cells which are electrically silent, although structurally intact and therefore are considered salvageable. The penumbral region has been identified as an area where ischaemic damage could be reduced and therefore the clinical outcome of stroke improved (Heiss, 2000) (Figure 2).

1.2.3.1 Ischaemic cascade

The occlusion of a blood vessel in the brain leads to a decrease in the blood flow, reduced availability of oxygen and glucose, and ultimately cell death (Martin et al., 1994). Immediately after the time of occlusion an ischaemic cascade is set in motion, with a variety of processes occurring in a temporally distinct fashion. Cell death initially occurs due to the lack of energy supply to the brain tissue. The ionic balance of neurones is disrupted due to the lack of adenosine triphosphate (ATP) available for ATP-dependent ion channels and thus a wave of depolarisation is created. This leads to the release of the excitatory neurotransmitter glutamate which binds to both N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors (Monaghan et al., 1989). This facilitates the release of Ca\(^{2+}\) and Na\(^{2+}\) into cells which initiates a cascade of detrimental downstream processes. A self propagating cycle of depolarisation is created resulting in excitotoxicity and
further enhancement of the ischaemic damage. Excitotoxicity can lead to the development of oxidative stress where reactive oxygen species such as super oxide anions (O$_2^-$) can no longer be neutralised within cells. Oxidative stress can initiate cell damage through the disruption of cell membranes and also by increasing vascular permeability (Allen and Bayraktutan, 2009). These can have severe consequences on the integrity of the blood brain barrier (BBB) (Figure 2).

2. Pattern of injury following cerebral ischaemia and mechanisms of cell death. A) Following the occlusion of a vessel, a distinct pattern of injury forms with an unsalvageable core adjacent to the blockage site. Surrounding this core is the penumbra which, although damaged is still considered salvageable. B) Ischaemia induces a state of energy failure which triggers downstream mechanisms leading to cell death and ultimately permanent cerebral damage.

1.2.3.2 BBB

The BBB is unique to the brain and acts to prevent free entry of molecules that could be harmful. The BBB is the reason why the brain was once considered immune privileged as it was thought that nothing could cross through it however this has since been disproved and it is now recognised that molecules smaller than 400Da can diffuse through the BBB (Pardridge, 2005). The BBB consists of endothelial cells and adjacent astrocytes, basement membrane, pericytes and neurones (Abbott et al., 2010). These are all part of a neurovascular unit which allows the entry of nutrients however it excludes any molecules which may be
toxic to the brain (Abbott et al., 2010). A complex network of tight junctions, coupled with a lack of fenestrae and low pinocytic activity prevents transcellular passage of molecules into the brain (Engelhardt and Sorokin, 2009). Damage to the BBB can occur by a number of methods including cell death and also degradation by enzymes, which can be released by immune cells arriving at a site of injury. Once a breakdown of the BBB occurs, bloodborne molecules and immune cells can freely access the brain. The activation of resident glia and entry of immune cell initiate a complex inflammatory response within the brain.

1.3 Inflammation

The stages involved in acute inflammation were first described around 2000 years ago by Celcius as calor, rubor, tumor and dolor. These have been translated in recent times to the cardinal symptoms we recognise as heat, redness, swelling and pain, which are associated with loss of function. The inflammatory response is induced in response to harmful exogenous stimuli such as pathogens and endogenous stimuli such as damaged cells. Sterile inflammation is induced by harmful endogenous stimuli and can initiate the inflammatory response in a similar way to exogenous pathogens. Sterile inflammation can be triggered by many factors including blood, mitochondria and oxidised lipids and is associated with a dysregulation in homeostasis and disease (Rock et al., 2010). The inflammatory response is an important protective mechanism as it facilitates the removal of harmful stimuli and promotes healing. The cellular inflammatory response can be divided into the innate and the adaptive immune responses. The cellular components of these all arise from pluripotent stem cells in the bone marrow. From there two particular lineages arise, the myeloid lineage (which governs the innate immune response but contributes to adaptive immune responses as an effector), and the lymphoid lineage (which initiates and controls adaptive immune responses). The major cellular responses involved in these are discussed below.

1.3.1 Innate immune response

The innate immune response is the first line of host defence and is induced in response to the identification of foreign molecules. The innate immune response is not affected by previous exposure to antigens and does not fundamentally alter over a lifetime. The major stages of the innate immune response include phagocytosis and activation of the complement system, which involves the propagation of the immune response and the killing
of microbes (Sjoberg et al., 2009). Phagocytes are the primary cells responsible for phagocytosis which involves the engulfing and destroying of invading pathogens and also in the release of inflammatory molecules. Phagocytes differentiate between host and non-host molecules but do not mount a specific response to individual infections. Phagocytes can be divided into two categories, the mononuclear phagocytes, monocytes and macrophages, and polymorphonuclear, granulocytes such as neutrophils. Mononuclear phagocytes can originate from monocytes which differentiate into both macrophages and immature dendritic cells (Yona and Jung, 2010). They are present both in the circulation and as resident macrophage cells in specific tissues such as Kupffer cells in the liver and microglial cells in the brain (Kreutzberg, 1996; Naito et al., 1997). Their specific role is in the engulfing and destroying pathogens. Immature dendritic cells can differentiate into mature dendritic cells following phagocytosis and travel to the lymph nodes where they serve as antigen presenting cells to activate the adaptive immune response (Savina and Amigorena, 2007). Neutrophils are the principle polymorphonuclear granulocyte and account for 95% of the total granulocyte population. These cells function both in phagocytosis and also in the release of inflammatory molecules which can recruit further phagocytes and also activate the adaptive immune response (Dale et al., 2008). There are other, non myeloid elements of the innate immune system, such as NK cells, which play a key role in defence against tumours or viruses (Shi et al., 2011).

1.3.2 Adaptive immune response

The adaptive immune response is tailored to the specific stimuli encountered, whether that is a specific pathogen or a host inflammatory molecule. The key element of the adaptive response is immunological memory. Lymphocytes, namely T and B lymphocytes are the major cell types involved in the adaptive immune response. Lymphocytes respond to antigens through specific receptors found on their surface. B lymphocytes carry antibodies (B cell receptor) to specific antigens on their surface and when detected, they differentiate into plasma cells which can then secrete vast quantities of these antibodies (Kelsoe, 1994). These antibodies can then bind directly to pathogens and facilitate their clearance from the body. Naive T lymphocytes travel from the blood to the lymphoid tissue where they engage with dendritic cells (Guermonprez et al., 2002). Dendritic cells of the innate immune response present antigens on their surface to T lymphocytes and facilitate their differentiation (Guermonprez et al., 2002). There are two major types of T lymphocytes, T helper (T\textsubscript{H}) and T cytotoxic (T\textsubscript{C}). T\textsubscript{H} cells are involved in the activation of other immune cells including B
lymphocytes, macrophages and \( T_C \) cells. \( T_C \) cells however respond to activation by antigens and release cytotoxins which can destroy infected cells and therefore clear pathogens. The adaptive immune response also facilitates the generation of both B and T lymphocyte memory cells which are stored in peripheral organs and can be mobilised quickly if the same pathogen presents.

It is possible to distinguish the different types of immune cells stated above through the specific markers on their surface. Each cell expresses a different combination of cluster of differentiation (CD) epitopes in the plasma membrane (Table 1) and using a combination of antibodies, certain populations and subpopulations of each individual cell type can be identified through flow cytometry.

<table>
<thead>
<tr>
<th></th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD11b</th>
<th>CD19</th>
<th>MHCI</th>
<th>CD11c</th>
<th>CD14</th>
</tr>
</thead>
<tbody>
<tr>
<td>All T cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T cells (helper)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T cells (cytotoxic)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Macrophages</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Monocytes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1. CD markers expressed on each immune cell type. The above table is a simplified model of CD antigen expression to discriminate the main populations used in this thesis. Each immune cell expresses a different combination of CD markers and when using a combination of antibodies against these markers for flow cytometry, it is possible to determine each individual cell population.

As well as the cellular inflammatory response, a number of molecular inflammatory mediators such as cytokines and chemokines have a pivotal role in the inflammatory response.
1.3.3 Cytokines and Chemokines

Cytokines are a large family of inflammatory molecules that are produced in response to infection and injury. There are a number of different cytokine groups including interleukins (IL), interferons (IFN), tumour necrosis factors (TNF) and growth factors. Chemokines are another family of cytokines and act as chemo-attractant proteins. These proteins bind to receptors found on immune cells and cause a recruitment of these cells in a concentration dependent manner. The actions of the cytokines most relevant to this thesis are described in Table 2.

<table>
<thead>
<tr>
<th>a) Cytokines</th>
<th>Family</th>
<th>Member</th>
<th>Principal effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukins (IL)</td>
<td>IL-1</td>
<td>Wide variety of biological functions on many different cell types, including lymphocyte activation, macrophage activation, increased leukocyte-endothelium adhesion, pyrexia, induction of the APN, neutrophil release</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>Regulates lymphocyte functions; affects hematopoiesis and induces APR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-10</td>
<td>Inhibition of proinflammatory cytokine synthesis and T-cell release</td>
<td></td>
</tr>
<tr>
<td>Interleukin-17 family (IL-17)</td>
<td>IL-17a</td>
<td>Induces IL-6, IL-8 and G-CSF (neutrophils) and APR, role in autoimmunity</td>
<td></td>
</tr>
<tr>
<td>Interferons (IFN)</td>
<td>IFN-γ</td>
<td>Affects activation, growth and differentiation of lymphocytes, macrophages and NK cells, up-regulates MHC expression, antiviral and anti-proliferative</td>
<td></td>
</tr>
<tr>
<td>Tumour necrosis factor (TNF)</td>
<td>TNF-α</td>
<td>Many functions, including stimulating the growth and differentiation of multiple cell types, promoting angiogenesis and thrombotic processes. Cytotoxic to most cells</td>
<td></td>
</tr>
<tr>
<td>Colony-stimulating factors (CSF)</td>
<td>Granulocyte (G)-CSF</td>
<td>Growth and differentiation of neutrophils</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>b) Chemokines</th>
<th>Sub-family</th>
<th>Member</th>
<th>Target cell for chemotaxis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>CCL-2</td>
<td>Monocytes, T cells, basophils, NK cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCL-5</td>
<td>Tcells, eosinophils, basophils, NK cells, dendritic cells</td>
<td></td>
</tr>
<tr>
<td>CXC</td>
<td>CXCL-2</td>
<td>Neutrophils, endothelial cells</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Cytokine and Chemokine mediators of inflammation. A) Family, members and the principle effects of those cytokines relevant to this thesis. B) Sub-family, members and target cell type of those chemokines relevant to this thesis. Table adapted from the thesis of Katie Chapman ‘Peripheral inflammation after experimental stroke’ (2009).

1.3.4 Inflammation and stroke

Many risk factors for stroke are associated with an elevated systemic inflammatory profile, shown both in clinical and preclinical studies, largely by elevations in circulating inflammatory mediators. In patients, circulating levels of IL-6 have been shown to be increased in disease states such as atherosclerosis and obesity as have levels of CRP
Animal models of these risk factors also show an increase in a diverse range of inflammatory molecules including, the acute phase protein serum amyloid A (SAA), IL-6 and KC (Vgontzas et al., 1997; Visser et al., 1999). This raised level of systemic inflammation has been shown to cause an increase in the development of inflammatory conditions such as atherosclerosis. Administration of the inflammatory molecule SAA to atherosclerosis prone mice caused an increase in the development of atherosclerosis which was associated with an increase in the recruitment and accumulation of macrophages (Dong et al., 2011). The higher level of systemic inflammation is thought to have a detrimental impact on post stroke outcome and has therefore been identified as a possible link between risk factors and subsequent occurrence of stroke. Systemic inflammatory markers have also been shown to play a key role in the development of injury post-ischemia, although this is not the primary focus of this project (for review see (Planas et al., 2006)). The mechanism by which this raised level of inflammation causes increased stroke risk however is undetermined.

Clinical studies of the inflammatory burden of stroke centre mainly on the circulating levels of inflammatory mediators and the immune cell profile, since access to cerebrospinal fluid and brain tissue is difficult. Several cytokines and chemokines expressed by peripheral immune cells can also be expressed by resident and or invading cells in the brain. Therefore to fully understand mechanisms by which systemic inflammation can impact upon the brain and increase stroke risk, researchers utilise appropriate animal models.

Ischaemia causes an activation of resident microglial cells in the brain (Figure 3). During activation they are capable of producing a number of different cytokines such as IL-1, IL-3, IL-6, IL-10 and TNFα (Hanisch, 2002), and also have a role in phagocytosis. They migrate into the ischaemic region and here they have been shown to proliferate (Denes et al., 2007). The role of microglial cells in the progression of damage in stroke is conflicting with studies showing both neuroprotective and neurotoxic effects. Studies in which proliferating microglial have been abolished show a worse outcome following ischemia (Lalancette-Hebert et al., 2007). Whereas administration of tetracyclines, inhibitors of microglial cells, were found to protect hippocampal neurones following global ischaemia (Yrjanheikki et al., 1998). In the ischaemic brain the phagocytosis of invading neutrophils by microglial cells was also found to be neuroprotective (Neumann et al., 2008). Further work is therefore needed to determine the specific role microglial cells play in the progression of damage and it is likely that the type of damage induced is pivotal in this determination.
Astrocytes are another type of glial cell found in the brain and like microglial cells, they have a role in normal homeostasis, they become activated following ischaemia (Figure 3). Following activation, astrocytes produce free radicals (Thornton et al., 2006), cytokines such as IL-1, IL-6 and TNFα (Gabryel and Trzeciak, 2001) and proteases such as matrix metalloproteinases (MMPs) which contribute to the breakdown of the BBB.

The integrity of the BBB can be compromised following ischaemia through for example oxidative stress and protease actions allowing entry of peripheral immune cells (Figure 3). Leukocytes, namely neutrophils, are the first cells to arrive to the ischaemic region and their levels have been shown to correlate with infarct volume (Buck et al., 2008). These have been shown to release free radicals and enzymes, such as MMPs which cause a further breakdown in the BBB and a worsened outcome following stroke (Romanic et al., 1998). Neutrophils can also adhere to endothelial cells and thus create a physical barrier within vessels following reperfusion and induce the ‘no-reflow’ phenomenon (del Zoppo and Hallenbeck, 2000).

Endothelial cells, which form part of the BBB, are a key interface between the periphery and the brain parenchyma (Abbott et al., 2010). These cells can also become activated in response to ischaemia and in turn release inflammatory cytokines and chemokines. Adhesion molecules such as, vascular adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1), are expressed on the surface of activated endothelial cells (Augustin et al., 1994) (Figure 3). Adhesion molecules play a key role in the infiltration of immune cells across the BBB. A number of different adhesion molecules are involved in the slowing, rolling, tethering, tight adhesion, crawling and finally transendothelial migration across the BBB (Hogg and Berlin, 1995). Neutralisation of adhesion molecule pathways causes a reduction in the damage following stroke, highlighting the central role of adhesion molecules in the inflammatory response to stroke (Vemuganti et al., 2004). However clinical trials aimed at reducing adhesion molecules were unsuccessful in attenuating damage associated with stroke (Sherman et al., 2001).
Figure 3. Neuroinflammatory changes in response to stroke. During ischaemic stroke the area supplied by the occluded vessel is starved of oxygen. This results in a large inflammatory response in the occluded area. Platelets in the vessel become activated. The endothelial cells of the affected vessel express adhesion molecules and firstly neutrophils are recruited. These release a number of different inflammatory molecules such as chemokines, cytokines and also enzymes capable of degrading the BBB. Neurones starved of oxygen begin to die and further inflammatory cells are recruited. Microglial cells and astrocytes become activated and migrate into the affected area.

Adaptive immune cells have also been implicated in the progression of injury. Severe combined immunodeficiency (SCID) mice, which lack both B and T lymphocytes show a reduction in infarct volume following cerebral ischaemia (Hurn et al., 2007). However mice deficient in only B lymphocytes showed no reduction in infarct volume (Yilmaz et al., 2006). T lymphocytes have been implicated in the latter stages of damage following cerebral ischaemia (Shichita et al., 2009) and mice deficient in T regulatory lymphocytes have been shown to have increased infarct volumes following ischaemia (Liesz et al., 2009).
Inflammatory cytokines such as; IL-1, TNFα and IL-6 are actively produced in the brain following stroke. Of these cytokines, IL-1 has been identified as a primary pro-inflammatory cytokine with multiple detrimental effects.

1.3.5 Interleukin-1

The IL-1 family of cytokines are key pro-inflammatory mediators which have been widely studied in relation to disease and development. There are eleven members of the IL-1 family of which; interleukin-1α (IL-1α), interleukin-1β (IL-1β) and interleukin-1 receptor antagonist (IL-1Ra), are the most well known (Dinarello, 2011). IL-1α and IL-1β are two biologically active forms of IL-1 and are both produced in a pro form (Luheshi et al., 2009). IL-1α is biologically active in both the pro and cleaved form whereas IL-1β is active only following proteolytic cleavage by caspase-1 (Mosley et al., 1987). These all have the same affinity for the interleukin-1 receptor-1 (IL-1R1) found on IL-1 responsive cells (Dower et al., 1986). Following binding to the IL-1R1, an IL-1R1/α/β complex is formed which triggers the binding of the IL-1R accessory protein to bind to the preformed complex Wesche et al., 1997). MyD88 is recruited to this complex and IL-1R associated kinases (IRAK’s) become phosphorylated (Weber et al., 2010). The transcription factor NF-κB is inhibited by IRAK’s, however following phosphorylation this no longer happens and therefore transcription of inflammatory molecules, such as CXCL1 and IL-6, occurs (Weber et al., 2010). CXCL1 has a role in chemotaxis especially in the recruitment of neutrophils to a site of injury. IL-6’s role is both in regulation of lymphocyte function and the induction of APR. These inflammatory molecules therefore elicit the actions of IL-1 such as induction of fever, regulation of the immune response and a key role in the pathogenesis of disease. IL-1Ra is an endogenous ligand of IL-1R1 and it functions to limit the actions of IL-1 and has thus been seen as a key research tool (Figure 4) (Dinarello, 2011).
Inflammation and has been negative bacterial infection (Cartmell et al., 1
1α, proIL-1α and IL-1β bind to the IL-1R1. This ischaemic damage was reduced by 60% after co administration of IL-1Ra thus implicating IL-1 as a major mediator of systemic inflammation (McColl et al., 2007). IL-1 plays a key role in inflammation and the administration of IL-1β elicited a similar effect to an LPS challenge. The gene encoding IL-1β is up regulated in the brain during LPS systemic inflammation in greater quantities than its counter regulatory cytokines (IL-10, IL-13 and IL-1Ra)(Wong et al., 1996). Knockout animals for both IL-1α and IL-1β show a marked reduction in infarct volume, demonstrating a key role in ischaemic injury (Boutin et al., 2001). IL-1β is also activated in the brain vasculature during systemic inflammation (Wong et al., 1996). Systemic administration of IL-1β has therefore been utilised in

![Image of IL-1 signalling](image-url)
experimental studies to mimic systemic inflammation, in order to gain a greater understanding of how it may contribute to stroke risk. This systemic challenge prior to MCAo was also found to produce a sustained increase in the disruption of the BBB (McColl et al., 2007), with an associated increase in neutrophil infiltration. Furthermore the neutrophil-derived enzyme, MMP-9 was found to be responsible for a decrease in the levels of claudin-5 (McColl et al., 2008a), a tight junction protein responsible for maintaining the integrity of the BBB, in a sustained fashion contrary to the biphasic BBB opening seen in the absence of systemic inflammation (Rosenberg et al., 1998). The increases in MMP-9, released from degranulating neutrophils (Opdenakker et al., 2001), occurs possibly as a result of greater interactions with degranulation stimuli such as cytokines, or an increased sensitivity to these stimuli. A role of systemic inflammation in the exacerbation of stroke injury is therefore outlined (Figure 5).
Figure 5. Involvement of IL-1 in systemic inflammation, elevated stroke risk and outcome. Infection, injury and other alterations in homeostasis are thought to induce raised levels of IL-1. IL-1 can then contribute to a raised level of systemic inflammation which, if left untreated can lead to an increase in chronic inflammatory diseases. Chronic inflammatory diseases such as atherosclerosis are associated with a further raised level of systemic inflammation, including vascular activation, leukocyte activation and increased levels of proinflammatory cytokines. This raised level of systemic inflammation can then induce neuroinflammation prior to ischaemic events such as glial activation and vascular activation. The chronic inflammatory diseases and raised level of neuroinflammation can prime the brain and lead to an exacerbation of damage and a worsening outcome if ischaemia occurred. Image taken from (Denes et al., 2011).

Risk factors for stroke such as atherosclerosis, diabetes and obesity have all been associated with a raised inflammatory profile and animal models of these risk factors also show a raised level of systemic inflammation. Interestingly animal models of risk factors for stroke such as the atherosclerotic ApoE<sup>-/-</sup> mouse (Horsburgh et al., 1999), the diabetic db/db
mouse (Vannucci et al., 2001) and the obese ob/ob mouse (Terao et al., 2008), show increased neuronal damage in response to cerebral ischaemia when compared with controls. Greater use of these models by researchers will allow the contribution of preceding conditions to stroke pathology to be studied in more detail and lead to the development of more effective treatments.

1.4 Stroke risk factors

Stroke patients rarely present without preceding conditions such as atherosclerosis, diabetes, obesity, hypertension and infection. These conditions are now considered as risk factors for stroke and increasing interest has focused on interlinking mechanisms by which they increase stroke risk, especially as there is much overlap between factors, with the development of one inevitably leading to the development of another.

1.4.1 Atherosclerosis

Atherosclerosis is a major contributor to stroke due to thromboembolisms arising from the rupture and detachment of vascular plaques (Ohira et al., 2006). The pathogenesis of atherosclerosis and formation of vulnerable plaques occurs in stages. The first stage involves activation of endothelial cells which show an increase in chemokine production, such as chemokine ligand 5 (CCL5), and an up-regulation of molecules that promote the adhesion of neutrophils and monocytes such as VCAM-1 and ICAM-1 (Gawaz et al., 2005). An increased release of chemottractant proteins such as monocyte chemoattractant protein-1 (MCP-1) and CCL5 is also observed, leading to an increased recruitment of monocytes and other inflammatory cells (Piga et al., 2007). Cells passing close to the luminal surface of the endothelium begin to slow and can tether to the expressed adhesion molecules. Following this they roll across the surface of the endothelium and then tightly adhere through tethering to further adhesion molecules. After this, tight adhesion cells migrate across the endothelium and transmigrate to the intimal layer, either intracellularly or through the spaces between cells. A thickening of the intima also occurs, due to an increase in the number of smooth muscle cells migrating to the luminal face (Figure 6). These cells synthesise proinflammatory cytokines such as TNF-α and IL-1. A fatty streak is then formed as lipids deposit into this thickened area (Figure 6). Recruited macrophages engulf low density lipoproteins (LDL) forming foam cells, which secrete proinflammatory mediators causing an increase in the expression of adhesion molecules on the surface of endothelial cells. Recruited T cells and
macrophages can then further infiltrate the developing plaque. A fibrous cap subsequently covers the lipid core, increasing the stability of the plaque (Figure 6).

![Diagram of atherosclerotic plaque development]

**Figure 6. Atherosclerotic plaque development.** Initiation of atherosclerosis occurs when adhesion molecules become expressed on the surface of endothelial cells. This expression allows the infiltration of immune cells and also the migration of smooth muscle cells. Lipids are deposited in the intimal layer forming a fatty streak. Recruited macrophages engulf deposited lipids and become foam cells which produce a number of inflammatory mediators. The intimal layer becomes thickened as an increasing number of cells are recruited to the plaque. An advanced lesion is characterised by a diverse accumulation of immune cells and smooth muscle cells and a necrotic core develops due to the ongoing inflammatory processes. A fibrous cap covers a stable advanced plaque and protects it from rupture. The fibrous cap can become compromised and is broken down by enzymes which cause the plaque to rupture. Once ruptured the contents of the plaque are leaked into the lumen of the affected vessel and platelets aggregate. As platelets aggregate and coagulate a thrombus is formed which can either occlude the affected vessel or travel through the vasculature to occlude a vessel supplying for example the brain.

Plaques can remain stable and asymptomatic within the vessel wall and cause no obvious health problems, however they can also continue to grow, eventually becoming symptomatic by obstructing the entire vessel or by rupturing (Hansson and Libby, 2006). Unstable plaques have been shown to contain a high level of infiltrating leukocytes capable
of producing enzymes and thrombogenic substances that can cause plaque rupture (Patel et al., 2008) resulting in a thromboembolism. A thrombus can travel through the vasculature until it becomes lodged within a vessel causing an ischaemic event in the supplied tissue. Inflammation promotes the development and also influences the stability of plaques. Injury to the vessel wall, whether due to atherosclerotic lesions or other mechanisms, causes the recruitment of platelets which are the key players in the formation of thrombi. In order to study atherosclerosis, a number of animal models have been developed which develop atherosclerotic plaques similar to humans.

1.4.2 Animal models of atherosclerosis

Prior to the development of transgenic mouse models, the study of atherosclerosis was mostly limited to large animals whose lesions were often small and developed over a long period of time (Jawien et al., 2004). In 1992 the Apolipoprotein E\(^{-/-}\) (ApoE\(^{-/-}\)) mouse was developed and provided a breakthrough in the study of atherosclerosis in mice (Plump et al., 1992). These mice were found to develop lesions in a similar fashion to humans (Nakashima et al., 1994). Initially experiments were carried out without the use of atherogenic diets as previously used diets were found to be toxic and it was thought that mice could not tolerate diets high in fat (Tepperman et al., 1964). However dilution of the fat content in these toxic diets provided an essential tool in the atherosclerosis field (Nishina et al., 1990). As well as the widely studied ApoE\(^{-/-}\) mouse a number of different transgenic animals have also been developed. These include the low density lipoprotein receptor knockout mouse, which also shows disrupted lipid transport (Getz and Reardon, 2006) and the JCR:LA corpulent rat, which has dysfunctional leptin signalling (Russell et al., 1998a).

1.4.2.1 Apolipoprotein E knockout mice

ApoE is a molecule found on the surface of lipoproteins (Mahley, 1988). Lipoproteins consist of triglycerides, cholesterol, phospholipids and apolipoprotein molecules (Mahley et al., 1984). They are synthesised on absorption from the intestine and are used to transport lipids to various cells of the body (Field and Mathur, 1995). The apolipoprotein molecule is responsible for the delivery of lipoproteins to specific cells through their interaction with their target receptors (Yamamoto et al., 2008). The ApoE molecule mediates the interaction of lipoproteins with the ApoE receptor of the liver (Hui et al., 1986). This interaction facilitates the uptake of lipoproteins by the liver and thus a regulation of plasma lipid levels (Plump et al., 1992).
Mice deficient in ApoE have been generated, through gene targeting in mouse embryonic stem cells, and have become an invaluable tool in the study of diet induced atherosclerosis. ApoE<sup>C/C</sup> mice develop severe hypercholesterolemia and atherosclerosis (Piedrahita et al., 1992). They show a marked increase in vascular pathology when fed a high fat diet compared to chow fed controls (Zhang et al., 1994). The pathology observed occurs in stages which parallels the human condition and therefore these animals provide an ideal basis for the study of atherosclerosis (Nakashima et al., 1994). ApoE<sup>C/C</sup> mice not fed an atherogenic diet develop end stage lesions between 35 and 40 weeks of age (Jawien 2004). They have been shown to have a raised level of systemic inflammation that extends beyond the atherosclerotic lesions (Lohmann et al., 2009) highlighting the potential for this disease to influence the whole organism. ApoE<sup>C/C</sup> mice have been shown to have an increased infarct volume in response to global and focal ischaemia when compared with controls (Plump et al., 1992; Laskowitz et al., 1997; Horsburgh et al., 1999). This observation could relate to the raised levels of systemic inflammation seen in not only these animals but also humans with a similar condition. ApoE<sup>C/C</sup> mice develop spontaneous atherosclerosis and show a number of detrimental inflammatory processes. However, this takes a long time and therefore pro-atherogenic diets have been developed which utilise the alterations in lipid transport in these mice and accelerate the formation of atherosclerosis (Plump et al., 1992).

### 1.4.2.1.1 Paigen Diet

The Paigen diet was developed by Beverly Paigen and contains 18.5% fat, 0.9% cholesterol, 0.5% cholate and 0.259% sodium (Nishina et al., 1990). Traditionally when this diet is used to induce atherosclerosis it is fed to mice aged between 8 and 12 weeks and is required to be fed for 8 weeks to develop advanced atherosclerotic lesions (Zhang et al., 1994). This acceleration in lesion development, with end stage lesions reached in approximately half the time of unfed mice is an attractive feature to many researchers. Hypercholesterolemia is induced severely and rapidly using the diet and this is attributed to the cholate content. It is thought that the presence of cholate causes an inhibition of cholesterol 7α hydroxylase (Ando et al., 2005). Cholesterol 7α hydroxylase is an enzyme required for the conversion of cholesterol to bile acid (Chiang, 2002). Thus in its absence, a higher level of cholesterol is available to a developing plaque. In the mouse this diet has been shown to exacerbate changes associated with atherosclerosis, such as increased expression of adhesion molecules, increased recruitment of multinucleated giant cells (Samokhin et al.,
2010), increased release of proinflammatory cytokines (Stokes et al., 2003) and increased platelet-leukocyte interactions (Stokes et al., 2006).

1.4.2.1.2 Western Diet

Of the two diets described here, the western diet is the most widely studied. The newer western diet was developed to more closely mimic the standard American diet. It differs from the Paigen diet in both its fat content and also its absence of cholate (21% fat, 0.15% cholesterol and 0% cholic acid). In order to develop atherosclerotic lesions comparable with Paigen diet fed animals, it is necessary for the diet to be used for 15 weeks. The western diet is thought to act mainly by increasing the levels of lipids within the blood. The dysfunctional lipid transport seen in ApoE\(^{-/-}\) causes a further rise in the plasma lipid profile and this is 3 fold higher than animals fed a chow diet (Grimsditch 1999). Thus the higher level of circulating plasma lipid accelerates the risk of these animals developing atherosclerosis.

Few studies have been performed with the aim of deducing whether atherosclerotic ApoE\(^{-/-}\) mice have neuroinflammation prior to ischaemic events. In order to study the effects of co-morbidities on stroke outcome, it must first be determined whether these animals have existing neuroinflammatory changes. Observing any neuroinflammatory changes may help understand why these mice show increased infarct volumes following experimentally induced stroke (Horsburgh et al., 1999) and why patients with these co-morbidities have a raised risk of stroke.

1.4.2.2 Corpulent rats

A number of mouse models are available for the study of atherosclerosis and associated diseases however, the development of larger animal models such as the rat is beneficial. This is particularly advantageous in imaging studies where the resolution of the images obtained is not capable of detecting changes in small animals, such as mice. One such animal model is the corpulent rat which is obese, hyperlipidemic, and insulin resistant and also develops atherosclerosis (Russell et al., 1987; Russell et al., 1990; Russell et al., 1998a). The predecessor of this rat was first identified from breeding a spontaneously hypertensive female with a normotensive male Sprague Dawley rat. The resulting litter contained rats which developed severe obesity (Koletsky, 1973). Further breeding studies were performed and the gene responsible, the corpulent gene, was found to be inherited recessively, with homozygous normal (+/+)) rats being lean (Koletsky, 1973). Hansen et al incorporated this
gene into the LA/N inbred strain (LA/N-cp rat) and began back crossing heterozygous animals to eliminate non-corpulent genes (Michaelis et al., 1983). However, after the 5th generation of back crossing a breeding colony was established by Russell et al (JCR:LA/N-cp rat) (Russell et al., 1987). Interestingly the phenotype of these two colonies remained the same however the atherosclerotic phenotype was lost in the LA/N-cp rat and preserved in the JCR:LA/N-cp rat (Russell and Amy, 1986b, a). Since their development a number of studies have been performed on the JCR:LA/N-cp rat, as they provide an ideal model to study multifactorial diseases that are increasingly present in the human population. Obesity in these animals develops early and at 12 months of age they have a body mass twice that of controls (Russell et al., 1989). Levels of the obese gene (ob) in white adipose tissue were found to be ten times that of controls (Vydelingum et al., 1995). The development of obesity has also been linked to increased food intake and alterations in satiety hormone release (Parnell and Reimer, 2008). The vascular dysfunction, atherosclerosis and myocardial lesions have only been reported in males, which show markedly increased lipid and insulin levels compared with females (Russell et al., 1998a). Lowering of lipid levels in these rats did not lead to an attenuation of the vascular disease (Russell et al., 1991) however, treatment of the hyperinsulinemia caused a significant decrease in vascular disease (Russell et al., 1997). Atherosclerotic lesion development follows the same structure as humans, being initiated with smooth muscle cell dysfunction and ending with plaques which contain multiple immune cells including engulfing macrophages (Russell et al., 1998a). JCR:LA-cp rats do not show insulin resistance from birth but show declining insulin sensitivity from 6 weeks, which correlates with rising levels of both plasma and skeletal muscle triglyceride levels (Russell et al., 1998b). The livers of these rats are hyperactive and have been shown to contain twice the level of mRNA indicating a greater number of cells. Production of very low density lipoprotein (VLDL), which contains the highest amount of triglycerides are markedly increased in the liver accounting for the raised level of plasma triglycerides (Elam et al., 2001). Therefore the corpulent rat is under a constant state of inflammation and is considered a co-morbid animal, thus is an ideal research tool to understand the contribution of systemic inflammation as a risk factor for stroke.

Atherosclerosis, as described in section 1.3.1., is a highly inflammatory disease which involves multiple immune cell types. In order to study the more intricate mechanisms occurring during atherosclerosis, animal models such as those described, have been utilised.
A key proinflammatory molecular mediator, IL-1, has been identified as a contributor to all stages of plaque progression and this is described below.

1.4.3 Interleukin-1 and atherosclerosis

Atherogenesis involves a number of different processes with inflammatory actions being pivotal to its progression. The proinflammatory cytokine IL-1 has long been associated with the pathogenesis of atherosclerosis and is heavily implicated in its initiation. It is produced by cells such as macrophages, which are key in the development of early lesions, but it is also detected by cells of the endothelium (Netea et al., 1998). Application of IL-1β to endothelial cells causes an increase in the expression of the adhesion molecule VCAM-1. IL-1α derived from activated platelets was also found to cause an increase in the expression of adhesion molecules (Thornton et al., 2010). An increased level of smooth muscle cell migration and the release of pro-atherogenic molecules such as MCP-1 upon IL-1 treatment has been reported (Gawaz et al., 2002). Smooth muscle cell proliferation is a hallmark of atherogenesis and IL-1 causes an increase in this via the downstream release molecule IL-6 (Massberg et al., 2003). Following endothelial and smooth muscle cell dysfunction, immune cells are known to infiltrate into the intimal layer and IL-1 has been shown to increase the vascular permeability of these cells (Martin et al., 1988). Presence of IL-1β causes dose dependent retention of lipids in macrophages and thus a pro-atherogenic phenotype (Persson et al., 2008). IL-1 is thought to contribute to the ongoing progression of atherosclerosis by providing a constant inflammatory environment required to maintain a plaque. Rupture of the fibrous cap covering atherosclerosis plaques is a known source of emboli responsible for ischaemic diseases, such as stroke and myocardial infarction. IL-1 has also been shown to have a role in this pivotal stage of the disease. It is thought to induce an increase in the secretion of MMPs that are capable of degrading the plaque (Mountain et al., 2007). As well as studying the role of IL-1 in the progression of the disease, scientific tools such as antibodies against IL-1, the endogenous antagonist IL-1Ra and transgenic animals, have been used to see how blockade of the IL-1 system affects the progression of disease. Chi et al showed that the mechanism of plaque initiation and progression was related to IL-1 signalling. They found a significant decrease in the development of plaques in Apo E<sup>−/−</sup> interleukin-1 receptor<sup>−/−</sup> animals (Apo E<sup>−/−</sup>/IL-1R1<sup>−/−</sup>) (Chi et al., 2004). ApoE<sup>−/−</sup> mice have also been crossed with both IL-1β<sup>−/−</sup> and IL-1α<sup>−/−</sup> mice and when fed an atherogenic diet both these models show a reduced level of atherosclerosis, demonstrating the role of both forms of IL-1 in this diseases progression (Kamari et al., 2007). ApoE<sup>−/−</sup> mice administered IL-1Ra also
show a reduced level of atherosclerosis and the administration of an IL-1β antibody also had the same effect (Bhaskar et al., 2011). These data together highlight the prominent role of IL-1 in atherosclerosis and thus the importance of its study in this inflammatory disease. IL-1 is a known mediator in many diseases and its actions post stroke have been well studied, therefore this cytokine may provide an important link between systemic inflammation induced by atherosclerosis and stroke.

1.4.4 Diabetes, obesity and hypertension

Diabetes, obesity and hypertension have all been identified as risk factors for stroke (O'Donnell et al., 2010). Diabetes is associated with an inability to control glucose homeostasis and can be classified into three different types; type I diabetes, type II diabetes mellitus (acquired) and gestational diabetes. Type I diabetes is a congenital disorder causing immunological destruction of islet β cells and the prevention of insulin production (Atkinson and Eisenbarth, 2001). Type II diabetes can be caused by a dysfunction in β cells and a peripheral insulin resistance (DeFronzo, 1999). Gestational diabetes occurs in pregnancy and is caused by an inability to produce enough insulin for normal glucose homeostasis however this usually resolves after birth (Buchanan and Xiang, 2005). All types of diabetes cause hyperglycaemia which if left untreated can be fatal. Hyperglycaemia is associated with development of type II diabetes which can itself lead to an increased risk of stroke and also development of atherosclerosis (DeFronzo, 1999). A close relationship between diabetes and the development of atherosclerosis has been drawn in recent years and the increased likelihood of diabetics having strokes highlights this (Heidemann et al., 2009).

Mechanisms behind the relationship between atherosclerosis and diabetes are unclear, though a number of theories exist. Acute periods of hyperglycaemia are common in diabetic and non-diabetic patients. A study by Piga et al found that the exposure of aortic endothelial cells to 12 hours of hyperglycaemia caused an increase in the expression of VCAM-1 on the endothelial cells and a subsequent increase in binding of monocytes (Piga et al., 2007). It should also be noted that diabetic patients demonstrated dysfunctional endothelial cells, shown as an increase in the adhesion molecules VCAM-1 and ICAM-1 (Gomez et al., 2008). In studies focusing on the link between diabetes and stroke, it was noted that traditional risk factors for stroke such as hypertension, dyslipidemia, heart failure and previous myocardial infarction were indeed higher in those patients with diabetes (Stegmayr and Asplund, 1995). Diabetic patients also showed an increased level of circulating CRP, a biomarker for stroke.
risk. Type II diabetes is associated with alterations in blood coagulation factors and thus these diabetics are considered to be in a hypercoagulable state (Carr, 2001).

Obesity is now being recognised as a pandemic (Naser et al., 2006) which leads to varying health complications. It is associated with hyperlipidemia, hyperglycaemia, hypertension and cardiovascular disease such as stroke. Raised levels of lipids have been associated with an increase in the development of atherosclerosis, due to increased lipid accumulation in vessels. Obese patients have been shown to have an increased risk of stroke (11%) (Eeg-Olofsson et al., 2009). Obesity is also being recognised as an inflammatory disease with inflammatory mediators being released from adipose tissue (Dandona et al., 2004). Obese mice also show raised levels of neuroinflammation compared with controls and have a worsened outcome following stroke (McColl et al., 2010). These findings are linked to the raised circulating levels of inflammatory markers with increased levels of CRP and IL-6 being observed (Vgontzas et al., 1997; Visser et al., 1999). Links between obesity and hypertension are also well recognised (Kotchen, 2008).

Hypertension is thought to affect nearly a quarter of the world’s population (Kearney et al., 2005). It is considered a risk factor for many diseases including stroke. A 5mm Hg lowering of diastolic blood pressure has been shown to cause a 30-40% reduction in stroke risk (MacMahon et al., 1990). Prolonged hypertension has been shown both clinically and experimentally to contribute to the progression of atherosclerosis (Alexander, 1995). Endothelial cells can be severely damaged by persistent hypertension (Todd, 1992). Damaged endothelial cells of vessel walls are then susceptible to infiltration and adhesion of platelets and monocytes, leading to obstruction of vessels or thrombus formation. Hypertension also exerts shear stress on plaques and thus increases the likelihood of rupture. Small vessel disease can develop due to increased pressure on the point of origin of small perforating arteries, which increases the thickening of the intima, leading eventually to the occlusion of the lumen (Alexander, 1995).

Risk factors for stroke such as obesity, hypertension and diabetes are all associated with increased systemic inflammation. As these diseases become more common the population becomes increasingly morbid. When combined together a co-morbid state is reached, which is highly inflammatory and increases the risk of diseases such as stroke.

1.4.5. Age

The single greatest risk factor for stroke is age as there is an increasing accumulation of risk factors. Greater than 70% of strokes occur in people over 65 years old and the risk of
having a stroke doubles with every decade after the age of 45 (Kelly-Hayes, 2010). The increased risk of stroke with aging is attributed to the increased accumulation of risk factors such as atherosclerosis and hypertension. Atherosclerosis is a disease which is more prevalent in the aging population and more advanced lesions are found to be present (Bax et al., 2003). These advanced lesions are more susceptible to plaque rupture and thus these aging patients are more at risk of stroke. Hypertension risk also increases with age and 90% of people surviving post 80 years of age have a lifetime risk of hypertension (Vasan et al., 2002). Risk of infection is also increased with age and therefore an aging population accumulates more risk factors for stroke which accounts for the increased prevalence of stroke in the elderly.

1.4.6 Infection

There is mounting clinical evidence for the role of infection as an initiator of stroke. The greatest increase in stroke risk has been identified as 3 days following initial diagnosis and this risk of stroke falls gradually in the following weeks (Smeeth et al., 2004). A study of over 50,000 people found that stroke risk was increased by up to 44% following respiratory and urinary tract infections (Smeeth et al., 2004). The authors noted the relevance of the different sources of infection, namely respiratory and urinary tract, producing equal risk of stroke. Respiratory infections such as Chlamydia (C.) pneumoniae and Streptococcus (S.) pneumoniae are accountable for the greatest number of infection related strokes (Clayton et al., 2008). Infection is likely to raise the systemic inflammatory profile of the patients and thus it is possible that this is the common feature between stroke and infection. An interesting study in patients found that vaccination against influenza caused a 50% reduction in the risk of stroke (Grau et al., 2005). This again shows the contribution of infection to stroke risk and guides towards greater preventative methods. Unexpected seasonal variations have also been shown to be important in the incidence of stroke in patients (Hindfelt and Nilsson, 1977), with an increased prevalence in the winter possibly representing an increased likelihood of common infection in the colder months (Muhammad et al., 2011).

Infection without other risk factors for stroke, such as atherosclerosis, has alone been highlighted as a risk factor. This role is seen most clearly in young patients as these patients are without the risk factors associated with aging, such as atherosclerosis, and therefore are not considered co-morbid. Infection is a common feature among young patients who experience stroke (Lee et al., 2008). Clinical observations of this patient group found that preceding infections were significantly higher in young stroke patients compared with controls (Syrjanen et al., 1988). Although not the primary risk factor for stroke in paediatric
children, Salih et al found 17.3% of children had infectious disorders (Salih et al., 2006). Infection is strongly associated with inflammation and it is this inflammatory aspect alone which may drive young patients towards a stroke prone state.

The link between infection and stroke is not clear. Infection causes a rise in systemic inflammation with increases in circulating inflammatory mediators such as CRP. Rises in the levels of inflammation during infection could therefore drive towards a more stroke prone state. Infection may also contribute towards the initiation of stroke through atherosclerotic plaque rupture. Atherosclerosis is itself an inflammatory disorder and the combination of this inflammatory response with the inflammatory response following infection could result in a negative outcome such as stroke. LPS is found on the surface of gram-negative bacteria and therefore entry of these bacteria leads to release of IL-1 into the infected organism (Garcia-del Portillo et al., 1997). LPS is known to cause considerable systemic inflammation (Raetz et al., 1991) and also to have an impact on thrombosis and thus stroke risk. Alterations in haemodynamics are also seen in infection-related stroke patients, circulating levels of antithrombotic activated protein C (APC) being reduced while there are raised amounts of C4b-binding protein, an inhibitor of anti coagulation protein S (Macko et al., 1996). In line with these findings, a decreased ratio of active plasminogen activator to plasminogen activator inhibitor were found (Macko et al., 1996). Such changes lean towards an increased coagulation profile and thus a greater chance of thrombus formation. However, these findings are not consistent between reports and therefore they may represent largely localised procoagulatory events (Lindsberg and Grau, 2003).

As well as being a risk factor for stroke, bacterial infections such as *C. pneumoniae* and *Porphyromonas (P.) gingivalis*, a key oral bacteria, have been detected in various stages of atherosclerotic plaques, and have been shown to be involved in both the initiation and progression of atherosclerosis (Belland et al., 2004). Animals infected with *P. gingivalis* show increased activation of endothelial cells in the aortic arch, shown by increased expression of VCAM-1, ICAM-1 and also an increased recruitment of macrophages (Lalla et al., 2003). *C. Pneumonia* has been detected in macrophages and interestingly when human macrophages are exposed to *C. pneumoniae* they degenerate into foam cells (Kalayoglu and Byrne, 1998). This is an interesting observation as foam cells have been identified as a preliminary feature in the early stages of atherosclerotic lesions. Macrophages infected with *C. pneumoniae* have also been shown to have an increased rate of adhesion to endothelial cells compared with controls. This observation was attenuated in ICAM-1 knockout animals.
suggesting that the role of *C. pneumoniae* in atherosclerosis is not independent of traditional initiating features (Takaoka et al., 2008). This data indicates a role for bacterial infections in atherosclerosis however, the most common bacteria to induce pneumonia, a key risk factor for stroke, is *S. pneumoniae* (Lynch and Zhanel, 2009).

### 1.4.6.1 *Streptococcus pneumoniae*

*S. pneumoniae* is a clinically relevant infection and has been identified as the most prevalent cause of community acquired pneumonia in patients (Ruiz-Gonzalez et al., 1999; Woodhead, 2002), as well as the number one cause of bacterial meningitis (Thigpen et al., 2011). In order for meningitis to occur a high level of circulating bacterium are required, as well as colonisation of the nasopharynx (Gerber and Nau, 2010). As discussed infection, especially respiratory infections, are a major risk factor for stroke, therefore it is likely that *S. pneumoniae* is accountable for many of the infection-related stroke incidents. It is unclear whether infection increases the incidence of stroke or only contributes to impaired outcome. *S. pneumoniae* results in raised systemic inflammation through the interaction of the pathogen with the host, as shown by increased circulating markers such as TNFα and CRP (Calbo and Garau, 2011). The involvement of *S. pneumoniae* in atherosclerosis has been sparingly studied but it has been shown to cause increased endothelial cell dysfunction and thus might contribute to the earlier stages of plaque development (Banerjee et al., 2010). In atherosclerotic mice, a vaccination programme against *S. pneumoniae* caused a decrease in lesion formation (Binder et al., 2003). This was attributed to higher levels of IgM antibodies against oxidised LDL and plasma from these mice was capable of blocking the uptake of lipids by macrophage cells. This study highlights a possible role for *S. pneumoniae* in atherosclerosis and thus further study into its contribution to diseases such as atherosclerosis and stroke is needed.

The role of *S. pneumoniae* in neuroinflammation has been more widely studied as it is the greatest cause of bacterial meningitis. The greatest levels of neuroinflammation have been shown to occur in models where a high dose of bacteria is administered intravenously, allowing the bacteria into both the cerebrospinal fluid (CSF) and the parenchyma (Chiavolini et al., 2008). Intravenous administration of bacteria overcomes the need of the bacteria to disseminate from a colony however, this model is not as clinically relevant as this type of infection is not a common occurrence in the clinic. *S. pneumoniae* is known to cause raised levels of microglial activation (Kreutzberg, 1996), which is thought to be related to the presence of bacteria within the brain. Increased leukocyte infiltration, as with atherosclerosis,
can cause endothelial dysfunction as shown by the increased expression of adhesion cell markers (Kadioglu et al., 2011). *S. pneumoniae* is the greatest cause of pneumonia in patients and although the effect of other bacterial infections has been studied in atherosclerosis, the effect of *S. pneumoniae* infections on atherosclerosis is yet to be determined. Neuroinflammation has been shown to occur following bacterial meningitis caused by *S. pneumoniae* however, it is not known how neuroinflammation is affected by an infection which has not disseminated to the brain.
1.5 Summary

This project will focus on how systemic inflammation contributes to the risk of stroke. Many risk factors for stroke are associated with an elevated level of circulating inflammatory mediators and stroke rarely appears without these associated conditions. It is also clear from experimental studies that pre-treatment with inflammatory mediators such as IL-1β worsen outcome following cerebral ischaemia. The risk factors associated with stroke generally interplay with each other, with a strong association placed on increased atherosclerosis. This is applicable in many stroke cases, although further insight is needed into stroke in the absence of atherosclerosis. The mechanisms by which preceding risk factors directly impact on stroke outcome are far from understood. A large body of work has focused solely on the events post stroke and inflammatory mediators, such as IL-1β have been shown to be key contributors to ischaemic injury. Most risk factors for stroke are known to represent a chronic inflammatory burden. If left untreated this burden could increase with age and drive towards a more stroke prone state. A theoretical stroke threshold could exist which could represent, for example, rupture of an atherosclerotic plaque that once passed, would cause the initiation of stroke. Infection could therefore provide a state of transient high grade inflammation which, combined with the already present systemic inflammation could reach the theoretical threshold and thus cause a stroke (Figure 7). Peripheral pathologies such as atherosclerosis could also lead to a raised level of systemic inflammation which could cause this theoretical threshold to be breached thereby initiating an ischaemic event.

![Figure 7. Relationship between risk factors and stroke.](image)

Risk factors for stroke have been shown to be associated with inflammatory mediators thus producing an inflammatory burden. This burden may increase with age and push towards a more stroke prone state. Coupled to this is the extra burden that may be encountered due to peripheral pathologies. Thus windows of a high stroke prone state may develop accounting for the association between infection and stroke risk (McColl et al., 2008b).
1.6 Aims

The overall aim of this project was to determine how systemic inflammation influences the neuroinflammatory status of the brain. To address this aim the specific objectives were to:

- Determine whether central nervous system (CNS) changes occur in both animal models and patients with risk factors for stroke. 

Risk factors for stroke, such as atherosclerosis and obesity, are known to have an inflammatory component involving many different immune cells and increased systemic inflammation. However, it is unknown whether any inflammatory changes occur in the brain in response to raised systemic inflammation associated with risk factor diseases prior to an ischaemic event. To do this, neuroinflammation will be assessed in the atherosclerotic mouse, the corpulent rat and patients at risk of having a stroke. This study will also be used to highlight any observation which can be translated from rodents to humans and thus highlight the appropriateness of animal models used.

- Determine how neuroinflammatory changes induced in response to peripheral atherosclerosis are affected by the deletion of IL-1 signalling and whether an IL-1β antibody can alter levels of both peripheral atherosclerosis and neuroinflammation.

Administration of pro-inflammatory cytokine IL-1β prior to stroke is known to cause an exacerbation of lesion volume and the use of the endogenous ligand IL-1Ra reduces lesion volume. IL-1 is therefore known to be important in the neuroinflammatory outcomes following stroke however it is unknown how neuroinflammation induced in response to peripheral IL-1 is affected. To study this ApoE<sup>+/−</sup>/IL-1R<sup>+/−</sup> mice will be used and levels of neuroinflammation will be assessed. ApoE<sup>−/−</sup>/IL-1R<sup>−/−</sup> mice have reduced peripheral atherosclerotic pathology compared with ApoE<sup>−/−</sup> controls and the use of an IL-1β antibody also reduces peripheral pathology. However, it is not known whether the IL-1β antibody used in this thesis has similar effects and whether an IL-1β antibody can reduce neuroinflammation associated with peripheral atherosclerosis. To do this, ApoE<sup>−/−</sup> mice will be administered the IL-1β antibody from the initiation of the pro-atherogenic diet.
Establish whether a peripheral infection in atherosclerotic mice induces any cerebral ischaemic events and to determine if any inflammatory changes occur in both the periphery and the brain in response to peripheral infection.

Both infection and atherosclerosis are risk factors for stroke and both involve inflammatory mechanisms. How these inflammatory mechanisms interact in a mouse model is currently unknown. The atherosclerotic ApoE\(^{−/−}\) mouse fed a Paigen diet will be infected with S. pneumoniae when advanced lesions are formed. The mice will be assessed behaviourally for cerebral ischaemia. Neuroinflammation will be assessed and peripheral organs and blood will be examined for inflammatory changes in response to both atherosclerosis and infection.
1.7 Papers and contributions of authors

Each aim stated above represents one chapter/paper in this thesis and the titles of these are given below. Paper 1 is currently published and the details of this publication can be found below. Papers 2 and 3 represent final manuscripts which will be submitted for review to the journals stated below.

Paper 1
Brain inflammation is induced by co-morbidities and risk factors for stroke.

Paper 2
Interleukin-1 mediates cerebrovascular pathology induced by atherosclerosis
Adam Denes#, Caroline Drake#, Jing Stordy#, Janet Chamberlain, Barry W. McColl, Hermann Gram, David Crossman, Sheila Francis, Stuart M. Allan, Nancy J. Rothwell. To be submitted to Circulation

Paper 3
Systemic and brain inflammation in mice in response to combined risk factors for stroke
Caroline Drake, Adam Denes, Peter Warn and Stuart Allan. To be submitted to Journal of Neuroinflammation

1.7.1 Contributions of authors
C.D. carried out a large portion of the work for each paper and performed all analysis on the data contributed to the papers. C.D. had a major role in the writing of each paper and the specific contribution to each paper is outlined below.

P.J.T., M.T., A.V., J.R.S., S.H., R.F.G and J.C.R. contributed expertise/reagents/materials/analysis tools. C.D. performed all work for Figures 2, 3, 4, and 5, H.B. performed all work for Figure 1 and M.J. performed all work for human P.E.T scanning. All authors contributed to the writing and editing of the paper.

Paper 2 – C.D., A.D., S.A., N.J.R., S.F., H.G. and D.C. conceived and designed the experiments. A.D., S.A., N.J.R., S.F., J.S., H.G. and D.C. contributed expertise/reagents/materials/analysis tools. The contribution of the authors to the figures is as follows Figures 1 S.F., C.D. and A.D. Figures 2 and 4, C.D. Figures 5, 6 and 7, Figure 3 J.S.. All authors contributed to the writing and editing of the paper.

Paper 3 – C.D., A.D., S.A. and P.W. conceived and designed the experiments. A.D., S.A. and P.W. contributed expertise/reagents/materials/analysis tools. All experimental work and analysis was carried out by C.D. C.D. wrote the manuscript with contributions from A.D. and S.A.
Chapter 2

Paper 1

Brain inflammation is induced by co-morbidities and risk factors for stroke.


Brain inflammation is induced by co-morbidities and risk factors for stroke

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ABSTRACT

Chronic systemic inflammatory conditions, such as atherosclerosis, diabetes and obesity are associated with increased risk of stroke, which suggests that systemic inflammation may contribute to the development of stroke in humans. The hypothesis that systemic inflammation may induce brain pathology can be tested in animals, and this was the key objective of the present study. First, we assessed inflammatory changes in the brain in rodent models of chronic, systemic inflammation. PET imaging revealed increased microglia activation in the brain of JCR-LA (corpulent) rats, which develop atherosclerosis and obesity, compared to the control lean strain. Immunostaining against Iba1 confirmed reactive microgliosis in these animals. An atherogenic diet in apolipoprotein E knock-out (ApoE-/-) mice induced microglial activation in the brain parenchyma within 8 weeks and increased expression of vascular adhesion molecules. Focal lipid deposition and neuroinflammation in periventricular and cortical areas and profound recruitment of activated myeloid phagocytes, T cells and granulocytes into the choroid plexus were also observed. In a small, preliminary study, patients at risk of stroke (multiple risk factors for stroke, with chronically elevated C-reactive protein, but negative MRI for brain pathology) exhibited increased inflammation in the brain, as indicated by PET imaging. These findings show that brain inflammation occurs in animals, and tentatively in humans, harbouring risk factors for stroke associated with elevated systemic inflammation. Thus a “primed” inflammatory environment in the brain may exist in individuals at risk of stroke and this can be adequately recapitulated in appropriate co-morbid animal models.

1. Introduction

Clinical and experimental evidence implicates inflammation in multiple phases of stroke aetiology and pathology (Allan et al., 2005; Amor et al., 2010; Denes et al., 2010a,b; McColl et al., 2009; Muir et al., 2007). Several of the risk factors for stroke, such as atherosclerosis, hypertension and diabetes/obesity are triggered and/or propagated by dysregulated systemic inflammatory processes (Dandona et al., 2004; Ross, 1999; Savoia and Schiffrin, 2006). Markers of elevated systemic inflammation are associated with increased stroke risk and brain lesions detected by magnetic resonance imaging (MRI) (Fornage et al., 2008; van Dijk et al., 2005). Like other statins, rosuvastatin, has multiple anti-inflammatory properties. It reduces cerebrovascular events in patients without hyperlipidemia but with raised C-reactive protein (CRP) levels
(Ridker et al., 2008). Angiotensin-converting enzyme (ACE) inhibitors can lower median CRP levels and result in better long-term outcome in stroke patients, after controlling for confounding variables and concomitant treatments (Di Napoli and Papa, 2003). Similarly, aspirin or other anti-platelet treatments are used prophylactically in patients at risk, but it is still unclear whether their beneficial properties are due to anti-aggregation effects or to a combination of anti-platelet and anti-inflammatory effects (Franks et al., 2010).

Therefore, although inflammation-driven co-morbidities are common and aetiologically important in stroke patients, exactly how systemic inflammation contributes to risk of stroke and to other neurological conditions remains to be determined.

Despite the almost ubiquitous nature of co-morbidities preceding stroke, there has been a relative paucity of studies incorporating these in experimental stroke research. This may have contributed to the lack of successful translation for a number of potential stroke treatments identified in pre-clinical studies (Endres et al., 2008; Fisher et al., 2009). One reason for the failure of translation may be that underlying inflammation associated with atherosclerotic risk factors modifies the mechanisms of post-ischaemic brain damage, including the type, magnitude and kinetics of the damaging processes. In support of this, we and others have shown that the extent of brain injury is exacerbated, and mechanisms of damage altered and/or aggravated, when experimental stroke is induced in animals with hypertension, diabetes, obesity or acute/chronic infection/inflammation (Coyle, 1984; Denes et al., 2010a,b; McColl et al., 2007; Terao et al., 2008; Vannucci et al., 2001). However, it is unclear whether co-morbid stroke risk factors can drive brain inflammation and induce a ‘primed’ inflammatory state in the brain prior to a cerebrovascular event.

Here we undertook a translational study to determine if risk factors for stroke, which involve chronic systemic inflammation, also induce brain inflammation in rodents and humans. We show that brain inflammation is present in rats and mice harbouring systemic vascular and/or metabolic disease and that analogous changes may be present in patients with clinical risk factors and evidence of systemic inflammation, as indicated by a raised concentration of circulating CRP.

2. Materials and methods

2.1. Pre-clinical studies

These studies were performed on (JCR:LA-cp) (+/cp/cp) corpulent rats, which are obese, atherosclerotic and insulin resistant and ApoE-deficient (ApoE−/−) mice fed an atherogenic diet, which exhibit severe atherosclerosis.

Animals were allowed free access to food and water and were maintained under temperature, humidity and light-controlled conditions. All animal procedures adhered to the UK Animals (Scientific Procedures) Act (1986).

Corpulent and lean heterozygous control rats (+/?) obtained from an established breeding colony at The University of Alberta, Edmonton, Canada (Mangat et al., 2007); were subject to PET scanning using specific translocator protein (TSPO; formerly known as peripheral benzodiazepine receptor) radiotracers [18F]FDP-714, at 9 (average body weight; +?: 411 ± 14 g; cp/cp: 720 ± 22 g), 12 (+?: 438 ± 18 g; cp/cp: 918 ± 33 g) (n = 4 per group) and 15 months of age (+?: 452 ± 15 g, n = 4; cp/cp: 0.979 ± 0.054 kg, n = 3).

Experiments were carried out in male ApoE−/− (JAX 2052, Jackson Laboratories, USA) and C57BL/6 control mice (Jackson Laboratories, USA) bred in-house at the University of Sheffield. Mice aged 8 weeks were fed normal chow (4.3% fat, 0.02% cholesterol) or a high fat/high cholate (Paiogen; 18.5% fat, 0.9% cholesterol, 0.5% cholate, 0.26% sodium) diet (Special Diet Services, UK) for 8 weeks.

2.1.1. Positron emission tomography

Rats were anaesthetised by isoflurane inhalation (induction, 5%; maintenance, 2–2.5%) in oxygen. [18F]FDP-714, a specific tracer for the TSPO (Boutin et al., 2008; Chauveau et al., 2009) was synthesised (James et al., 2008), and injected intravenously in the tail vein as a bolus (10.8–19.8 MBq, 0.03–2.79 nmol). Respiration and temperature was monitored throughout using a pressure sensitive pad and rectal probe, Model 1025L interface and PC-SAM software (SA Instruments, NJ, USA). Body temperature was maintained at 37 ± 0.5 °C by use of a heating pad and the heating and fan module connected to the rectal probe via the interface and controlled by the PC-SAM software. Whole-body images were acquired in list-mode with a non-rotating 16-module quad-HIDAC PET camera (Oxford Positron Systems, UK) for 1 h (Hastings et al., 2007). The list-mode data were reconstructed directly into 5 min time-frame images (without resorting to histogramming) via the one-pass-list-mode-expectation maximisation (OPL-EM) algorithm (Reader et al., 2002) with one iteration of 16 sub-sets into images of dimensions 1202 (transaxially) × 240 (axially) with isotropic 1 mm3 voxels. Absolute calibration of the images was achieved by reference to a [22Na] source imaged in the field of view in each scan. This had been validated with a uniformly filled mouse-sized [18F] fluorodeoxyglucose PET phantom imaged over 2 h. Dynamic images were calibrated in kBq/cm3.

Images were segmented using the Local Means Analysis method and the organ mean Time Activity Curves were corrected for Partial Volume Effect using the Geometric Transfer Matrix (GTM) method with a selection of 20% of the organ voxels (GTM20) (Maroy et al., 2008a,b). The segmentation method extracts regions with homogeneous TACs, as required by the GTM20 method. The latter was designed to be more robust than the original GTM method to segmentation errors through the automated selection of adequate voxels in the segmented organs. Both methods were applied using the BrainVisa and Anatomist framework. For more accurate quantification and illustration purposes, PET images were co-registered with the rat MRI template (Schwarz et al., 2006), generously provided by GlaxoSmithKline (Verona, Italy). Automatic segmentation of PET images revealed 1–2 regions of interest (ROI) with different [18F]FDP-714 (low and high) uptake in the brain of both the lean and corpulent rats. These ROIs were used to compare the genotypes and the different ages.

To account for the differences (~2-fold) in body-weight between lean and corpulent rats, we expressed all uptake values as standardised uptake value (SUV) (i.e. percentage of injected dose per cubic centimetre corrected for body weight; SUVID/body). Until now the problem of comparing obese and lean animals or patients, and using SUV, has been mainly applied to [18F]fluorodeoxy-glucose PET imaging. However, considering the controversial literature on SUV, and the fact it has been reported that correcting for the absolute body-weight was likely to over-compensate for the difference (Boelaard, 2009; Keynes, 1995; Sugawara et al., 1999) we have used a slightly different approach. Indeed, the over-compensation of SUV is due to the fact that it assumes that the excess of weight mainly due to adipose tissue has the same metabolic activity than the rest of the body (Keyes, 1995; Sugawara et al., 1999), and therefore that corpulent rats have a metabolic activity twice that observed in lean controls. To the contrary, we considered that correcting for the lean body-weight was likely to under-compensate since it assumes that the excess of adipose tissue is completely inert (Keyes, 1995). Both assumptions being wrong, we decided to adjust the body-weight to calculate the SUV according to Kleiber laws (Kleiber, 1947), in which the metabolic activity is proportional to a factor equal to m0.74 (m being the body-weight in g of the animal).

2.1.2. Tissue processing

Under terminal anesthesia, mice and rats were perfused transcardially with saline followed by 4% paraformaldehyde...
(PFA; Sigma, UK). Brains were removed and postfixed in 4% PFA at 4 °C for 24 h. Brains were subjected to cryoprotection in phosphate-buffered saline containing 20% sucrose for 24 h. Five alternate sets of 20 μm (mice) or 30 μm (rats) thick coronal brain sections were cut on a sledge microtome (Bright series 8000; Bright Instruments, Huntingdon, UK). All sections were collected into an antifreeze solution (containing 30% ethylene glycol (Sigma, UK) and 20% glycerol (Fisher, UK) in phosphate-buffered saline) and stored at −20 °C until processing.

2.1.3. Immunohistochemistry

Immunohistochemistry was performed on free-floating brain sections. Endogenous peroxidise activity was blocked with 0.3% hydrogen peroxide (Sigma) in dH2O and sections were treated with 2% normal serum (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were incubated overnight in antibody diluent (0.1 M PBS + 0.3% Triton X-100, Sigma) using the following primary antibodies: goat anti-mouse VCAM-1 1:250 (R&D Systems, UK), goat anti-mouse ICAM-1 1:250 (R&D Systems, UK), goat anti-mouse Iba1 1:500 (Abcam, UK), rabbit anti-Iba1 (Wako Chemicals, Germany) and rat anti-mouse CD45 1:250 (Serotec, UK). Sections were then incubated in appropriate biotinylated secondary antibody for 1 h (rabbit anti-goat 1:1000 and rabbit anti-rat 1:750, Vector Laboratories, UK). Sections were then incubated in Vectastain ABC solution (Vector Laboratories, UK) and colour was developed by nickel enhanced diamino benzidine (50 mg/ml) incubation (Vector Laboratories, UK). Sections were mounted onto gelatin coated slides, dehydrated and coverslipped using Depex (Fisher, UK). Images were collected on an Axioscan colour CCD camera (Zeiss, Germany) upright microscope using 20× and 60× objectives and captured with a Coolsnop ES camera (Photometrics) through Axiosvison software (Zeiss, Germany).

2.1.4. Immunofluorescence

Double or triple immunofluorescence was performed on free-floating brain sections. After blocking in 2% normal donkey serum (Vector Laboratories) sections were incubated overnight at 4 °C in primary antibodies: rat anti-mouse CD45 1:200 (Serotec, UK), goat anti-mouse VCAM-1 1:250 (R&D Systems, UK), goat anti-mouse ICAM-1 1:250 (R&D Systems, UK), rat anti-CD3 (Serotec), goat anti-Iba1 (Abcam, UK), rabbit anti-Iba1 (Wako Chemicals, Germany) and rabbit anti-neutrophil serum (SJ), kindly provided by Drs. Daniel Anthony and Sandra Campbell, University of Oxford (Anthony et al., 1998). The antigens were visualised with the adequate fluorochrome-conjugated (Alexa 594 1:750 or Alexa 488 1:500, Molecular Probes) secondary donkey antisera or with biotinylated secondary antibodies followed by streptavidin Alexa 350 conjugate, for 2 h at room temperature. Sections were mounted onto gelatin-coated slides and cover-slipped Vectashield mounting medium containing diamidinophenylindole (Vector Laboratories, Burlingame, CA).

Images were collected on an Olympus BX51 upright microscope using 40× and 60× objectives and captured with a Coolsnap ES camera (Photometrics, UK) through MetaVue Software (Molecular Devices, UK). Specific band pass filter sets for DAPI, FITC and Texas red were used to prevent bleed through from one channel to the next.

2.1.5. Quantitative analysis

All quantitative analysis was performed under blinded conditions and confirmed by at least two independent researchers. VCAM-positive blood vessels were counted in three random fields of view for each section (typically 8–10) containing rostro-caudal cerebral cortex. A score for the whole brain was obtained by averaging individual counts and this was expressed as positive blood vessels per mm². Activated microglia were identified as showing: (1) increased Iba1 immunopositivity, (2) enlarged and/or amoeboid cell body, (3) complete or partial loss of thin, elongated processes. Round shaped, small Iba1-positive cells with leucocyte morphology were not counted. Regions analysed for microglial activation were also stained with mouse anti-rat CD68 (corpulent rats) and rat anti-mouse CD45 (mice) to assess the number of parenchymal macrophages and other leucocytes. Activated microglia were counted throughout the striatum and expressed as activated microglia per mm².

Fluorescently labelled CD45 positive cells were counted in two randomly selected fields of view of the caudal choroidal Plexus (−1.82 mm from Bregma) and the lateral ventricle (−1.58 mm from Bregma). The choroid plexus and ventricular ependyma were visualised by using VCAM immunofluorescence.

2.1.6. Histology

After CD45 immunohistochemistry (see above) sections were rinsed in dH2O and incubated in 60% v/v isopropanol/dH2O (Fischer, UK) for 2 min. Sections were transferred to Oil red O (ORO; Sigma, UK) (0.05% w/v ORO/99% isopropanol) for 15 min, rinsed in 60% v/v isopropanol, rinsed in dH2O and coverslipped with an aqueous glycerol jelly mount (7.7% w/v Gelatine (BDH, UK) and 54% glycerol in water). Haematoxylin & Eosin (H&E) staining was performed on mounted brain sections. Following staining sections were dehydrated and cover-slipped with Depex mounting medium.

2.1.7. Statistical analysis

Quantitative analysis of data was performed in a blinded manner. PET image quantifications were analysed using Mann–Whitney for comparison between lean and corpulent animals and for comparing 9 vs 15 and 9 vs 12 month age groups. Because the same group of animals was scanned at 12 and 15 months of age, a non-parametric paired Wilcoxon test was used to compare these two groups.

Quantitative data from immunohistochemical and immunofluorescence studies were analysed by one- or two-way analysis of variance (ANOVA) followed by post-hoc Bonferroni’s correction. All data are expressed as mean ± SD. Statistical significance is reported at the 0.05 level.

2.2. Clinical study

2.2.1. Patients

This small, preliminary study was undertaken to assess cerebral inflammation in humans with multiple risk factors for stroke, but no evidence of cerebral damage, in order to investigate the relevance of our experimental findings in a translational context. One hundred and twenty-one subjects were screened, and rigorous criteria were applied to identify patients at risk of stroke, while excluding patients with existing brain pathology. Subjects were deemed eligible if having multiple (three or more) risk factors for stroke, and/or established arterial disease (hypertension, dyslipidaemia, atrial fibrillation, left ventricular hypertrophy, ischaemic heart disease, diabetes mellitus, peripheral vascular disease, carotid disease and smoking), and CRP >3 mg/L on two separate occasions. All subjects underwent MRI scans to exclude any intracranial pathology, and subjects with a history of a previous cerebrovascular event were not involved in the study. MR scans were reviewed by neuroradiologists. Only four patients fulfilled all inclusion criteria and were subjected to PET imaging to assess microglial activation in the brain (see below). Age matched control participants were chosen on the basis of having two or fewer major vascular risk factors and plasma CRP ≤1 mg/L (see Table 1). All participants were also screened to exclude cognitive impairment and a
In ApoE⁻/⁻ mice fed Paigen diet lba1 immunohistochemistry revealed activated microglial cells (Fig. 2B). Microglia displaying thickened processes and increased levels of lba1 were observed in multiple brain regions such as the cerebral cortex, striatum, hypothalamus, periventricular areas and meninges. Chow or Paigen-diet fed C57BL/6 control mice and chow-fed ApoE⁻/⁻ mice lacked activated brain microglia.

### 3.3. Atherogenic mice develop vascular inflammation and leucocyte infiltration in the brain

C57BL/6 or ApoE⁻/⁻ mice fed a chow diet did not show elevated vascular ICAM or VCAM expression in the brain. A trend for increased vascular ICAM and VCAM expression was observed in C57BL/6 mice fed the Paigen diet (not significant). In contrast, ICAM and VCAM expression was significantly augmented in ApoE⁻/⁻ mice on the Paigen diet (Fig. 3A and B). Increased VCAM staining was present mainly on medium sized or large blood vessels in the cerebral cortex, striatum, thalamus and hippocampus. Quantitative analysis of VCAM immunohistochemistry revealed significantly stronger staining in Paigen fed groups compared to chow diet (P < 0.01, data not shown). Post-hoc comparison revealed significant differences between ApoE⁻/⁻ chow and Paigen fed animals (Fig. 3C), but not in C57BL/6 mice, indicating that diet-induced pro-inflammatory changes are augmented in ApoE⁻/⁻ mice.

We also investigated the possibility that diet-induced atherosclerosis was associated with leucocyte infiltration into the brain parenchyma and ventricles, using immunofluorescent staining of the common leucocyte antigen CD45. Microglial CD45 expression was relatively dim throughout the brain and was well discriminated from that of bright and round shaped or elongated leucocytes. Profound enrichment of ventricular leucocytes was found in ApoE⁻/⁻ mice fed with Paigen diet, and this was associated with increased VCAM expression in the choroid plexus (Fig. 4A). Invasion of the choroid plexus by CD45-positive cells was significantly elevated in ApoE⁻/⁻ animals on the Paigen diet compared to ApoE⁻/⁻ animals on normal diet, but this was not observed in C57BL/6 mice fed with Paigen diet (Fig. 4B). In Paigen-fed ApoE⁻/⁻ mice, CD45-positive cells were numerous in the choroid plexus of the lateral ventricles from the fimbria hippocampi to the caudal areas of the ventricle. Caudally, infiltration of ventricular-associated cells into the surrounding parenchyma was also observed in ApoE⁻/⁻ mice (Fig. 4C). The size of the lateral ventricle was not significantly different among experimental groups and no correlation between CD45-positive cells and ventricle size was found in individual mice. The choroid plexus was found to contain a number of different cell types including granulocytes (identified by an anti-neutrophil serum, 5J) and CD3-positive T cells (Fig. 4D). Granulocytes represented a large proportion of the cells and were uniformly distributed along the VCAM-positive areas of the choroid plexus, in partial overlap with T cells. Activated microglia/macrophages lined the walls of the caudal lateral ventricle, showed increased CD45 expression (Fig. 4E).

### 3.4. Atherogenic diet results in focal lipid deposition and inflammation in ApoE⁻/⁻ mice

In peripheral tissues, particularly in large blood vessels, ApoE⁻/⁻ mice develop atherosclerotic plaques, as identified by lipid deposition, leucocyte infiltration and vascular stenosis (Stoll and Bendszus, 2006; Zadelaar et al., 2007). However, it is not known whether such focal vascular pathologies appear in the brain in these animals or not. In 40% of the Paigen fed ApoE⁻/⁻ mice, focal pathologies were observed in the brain parenchyma (typically in the hypothalamus, near the third ventricle). Oil red staining identified blood vessel-associated lipid deposition (Fig. 5A and B).

### Table 1

Clinical study group characteristics.

<table>
<thead>
<tr>
<th>At risk subjects</th>
<th>Control participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex M:F</td>
<td>3:1</td>
</tr>
<tr>
<td>Number of risk factors</td>
<td>3–4</td>
</tr>
<tr>
<td>Mean CRP at screening</td>
<td>9.15 [2.99–13.26]</td>
</tr>
<tr>
<td>Mean interleukin-6 at screening</td>
<td>12.00 [1.98–33.40]</td>
</tr>
<tr>
<td>Mean CRP at PET range</td>
<td>11.93 [8.98–15.73]</td>
</tr>
<tr>
<td>Mean interleukin-6 at PET range</td>
<td>10.55 [3.70–25.00]</td>
</tr>
</tbody>
</table>

Table 1

Clinical study group characteristics.

- **At risk subjects**
  - Mean age in years: 63 [58–72] (n = 4)
  - Sex ratio: M:F = 3:1
  - Number of risk factors: 3–4
  - Mean interleukin-6 at screening: 12.00 [1.98–33.40]
  - Mean CRP at PET: 11.93 [8.98–15.73]
  - Mean interleukin-6 at PET: 10.55 [3.70–25.00]

- **Control participants**
  - Mean age in years: 64 [58–68] (n = 4)
  - Sex ratio: 1:3
  - Number of risk factors: 1–2
  - Mean CRP at screening: 0.76 [0.55–1.00]
  - Mean interleukin-6 at screening: 2.46 [1.10–3.61]
  - Mean CRP at PET: 1.56 [1.18–2.05]
  - Mean interleukin-6 at PET: 4.79 [1.00–8.08]

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telephone consultation was used to exclude symptoms of acute infection prior to PET scanning. All participants gave written informed consent.

### 2.2.2. Positron emission tomography

Participants underwent MRI scans on a 3 T Philips Achieva system using a T1 weighted inversion recovery SENSE sequence for co-registration of PET images and to exclude visible evidence of stroke. PET studies were performed on a high resolution research tomograph (CTI/Siemens). $^{11}$C-(R)-PK11195 (TSPO ligand) was used to assess microglial activation in the brain. Following a 6 min transmission scan, [11C](R)-PK11195 was injected as a slow bolus over 20 s and data were acquired during a 60 min emission scan. The injected radioactivity dose was 465 ± 121 MBq and radiochemical purity was always greater than 98.9%. The injected mass +53% in low and high uptake ROI respectively; and a supervised clustering algorithm was used to extract a reference tissue input function (Turkheimer et al., 2007). The study was approved by the local research and ethics committee.

### 3. Results

#### 3.1. PET imaging reveals neuroinflammation in cp/cp JCR-LA cp rats

There was no significant difference in microglial activation as determined by PET imaging between lean and corpulent rats at 9 months of age (Fig. 1A). By 12 months of age, microglial activation was increased significantly in the brains of the corpulent rats in the ROI with the lowest tracer uptake (+35%, Fig. 1B). $^{[18]$F}F}DA-714 uptake increased further in 15 month old animals (+32% and +53% in low and high uptake ROI respectively; Fig. 1C). We also observed a trend for an increase in neuroinflammation with age in both lean and corpulent animals, although this was significant only in the corpulent in the low uptake ROI when comparing 15 with 9 month old animals (+28%, P < 0.05, Fig. 1C). Although the $^{[18]$F}F}DA-714 uptake was increased by a similar magnitude (+29 to 33%, Fig. 1B and C) between 12 and 15 months in the corpulent rats, the differences were not significant. In reference organs, known to express high level of TSPO (heart, lungs and kidneys), there were no significant differences between lean and corpulent rats (Supplementary Fig. 1).

#### 3.2. Immunohistochemical evidence of microglial activation in rodents with peripheral disease

Immunohistochemistry revealed activated microglial cells in the brains of 15 month old corpulent rats (Fig. 2A). We found no activated microglial cells in the brains of corpulent rats aged 9 months or in heterozygous (lean) rats at any age (9–15 months) examined.

In ApoE⁻/⁻ mice fed Paigen diet lba1 immunohistochemistry revealed activated microglial cells (Fig. 2B). Microglia displaying thickened processes and increased levels of lba1 were observed in multiple brain regions such as the cerebral cortex, striatum, hypothalamus, periventricular areas and meninges. Chow or Paigen-diet fed C57BL/6 control mice and chow-fed ApoE⁻/⁻ mice lacked activated brain microglia.
accompanied by microglial activation and leucocyte recruitment (identified by H&E), as well as CD45 and Iba1 staining (Fig. 5C). VCAM expression was increased focally around lipid rich areas and also in the ipsilateral wall of the third ventricle, indicating ongoing inflammatory responses in the brain (Fig. 5D).

3.5. PET imaging: pilot study reveals neuroinflammation in human subjects with risk factors for stroke

Peripheral inflammatory markers increased in both groups of subjects between screening and time of PET but remained higher in the at risk group (Table 1). Visual inspection of the participants’ BPND maps revealed increased $[^{11}]$C(R)-PK11195 binding in three of the subjects with increased risk factors (Fig. 6). The distribution of the $[^{11}]$C(R)-PK11195 signal showed individual differences and was seen across neocortical areas and other brain regions, including the thalamus and brain stem. There was no evidence of raised $[^{11}]$C(R)-PK11195 binding in periventricular or deep white matter regions. There was no pattern of activity in a particular vascular territory, as one might see with established stroke. These preliminary results indicate that neocortical inflammation is present in the brain of subjects with chronic...
Fig. 2. Rodent models of atherosclerosis involve microglial activation in the brain. (A) Activated microglia as identified by increased Iba1 immunopositivity, thickened processes and irregular cell bodies were seen in the striatum of 15 month old corpulent rats, but not in 9 month old animals. Aged corpulent rats had a significantly increased number of activated microglia compared to young corpulent, or 15 month old heterozygous rats. (B) Activated, Iba1-positive microglia was numerous in ApoE^{-/-} mice fed a Paigen diet. Insets show representative images of microglial cells from the different groups of mice. Quantitative analysis revealed significantly more activated microglial cells in the striatum of ApoE^{-/-} mice fed a Paigen diet compared with ApoE^{+/-} mice fed chow diet. *P < 0.05. Scale bars: 200 and 10 μm (insets).

Fig. 3. Cerebrovascular activation occurs in the brain in association with peripheral atherosclerosis. Vascular activation was assessed in the cerebral cortex using immunostaining to the adhesion molecules (A) ICAM and (B) VCAM. Unlike mice fed a chow diet, mice fed a Paigen diet showed an increased number of ICAM and VCAM-positive blood vessels in the brain. (C) Quantitative analysis of VCAM-positive blood vessels in the cerebral cortex. Scale bars: 200 and 50 μm (inset).
systemic inflammation, which is consistent with our findings in rodents with risk factors for stroke.

4. Discussion

Here we show that major risk factors for stroke such as atherosclerosis, hyperlipidemia and obesity, which involve chronic systemic inflammation, are associated with brain inflammation in relevant animal models and in a small cohort of humans, in the absence of any cerebrovascular events. These data suggest that systemic inflammation can drive brain inflammation prior to stroke presentation, leading to a “primed” inflammatory environment in the brain.

We used PET imaging to identify microglial activation, because these cells are early responders to pathological changes in the CNS and microglial activation is a hallmark of multiple brain diseases in patients and rodent models (Hanisch and Kettenmann, 2007; Teeling and Perry, 2009). The advantage of assessing neuroinflammation by in vivo PET imaging in rodents is that these measurements are comparable with clinical imaging data, and is therefore highly translatable to clinical settings. Both [11C]PK11195 and [18F]DPA-714 bind to TSPO, but despite that [18F]DPA-714 has the advantages of better signal to noise ratio (Chauveau et al., 2009) and the longer half-life of [18F], which allow PET imaging of 2–3 animal per batch of tracer, [18F]DPA-714 is not yet available for clinical use in our facilities. Corpulent rats exhibited focal areas of microglial activation, as assessed by increased [18F]DPA-714 binding in vivo. Increased TSPO-ligand binding was observed in various brain areas, including periventricular regions and some subcortical and cortical regions in the corpulent rats (Fig. 1B and C). Imaging data correlated well with the immunohistochemistry findings, which revealed an increase in the number of activated microglial cells. In line with the experimental data, the presence of [11C](R)-PK11195 binding indicated neuroinflammation in subjects with multiple risk factors.

Microglial activation was also detected in several brain regions in atherosclerotic ApoE−/− mice, indicating that neuroinflammation is likely to be a common link among animal models of chronic systemic inflammatory diseases. Although the exact mechanism of microglial activation needs to be further investigated, such “priming” of microglia in response to peripheral inflammatory changes has important implications to multiple cerebrovascular diseases. It is now established that microglia primed by central neurodegeneration or amyloidosis respond more vigorously to subsequent systemic or central inflammatory insults. For example, in a murine...
model of prion disease, intracerebral or systemic LPS challenge induced augmented microglial activation and cytokine expression compared to control mice (Cunningham et al., 2005). Our data indicate that systemic influences are also capable of priming the inflammatory response of the brain. The presence of activated microglia and cerebrovascular inflammation may not only lead to irreversible neuroinflammatory alterations in the brain, but probably contribute to outcome if an ischaemic event occurs. Given that the vast majority of experimental stroke studies are undertaken in ‘normal’ animals with no underlying inflammation this might explain the lack of translation of potential treatments to the clinic.

We performed further characterisation of the vascular and cellular response in the brain of C57BL/6 and ApoE/−/− mice, to explore the possible effects of atherogenic diet on neuroinflammation. Although the atherogenic ‘Paigen’ diet alone reportedly induces inflammation in peripheral organs (Desai et al., 2008), we found significant vascular activation or enrichment of CD45-positive cells in the choroid plexus only in ApoE/−/− mice fed the Paigen diet, not in C57BL/6 mice. No sign of intraluminal plaques was observed in cerebral blood vessels in our study, which is in line with a report showing increased oxidative stress and endothelial dysfunction in cerebral arterioles in high-fat fed ApoE/−/− mice, but in the absence of atherosclerotic lesions (Kitayama et al., 2007).

Our results indicate that brain inflammation is associated with chronic systemic inflammation, and an atherogenic diet further augments this process. In Paigen-fed ApoE/−/− mice an increase in T lymphocytes in the choroid plexus at the areas of granulocyte recruitment was seen. A recent report in experimental autoimmune

Fig. 5. Focal pathological changes are present in the brain in response to peripheral atherosclerosis. Haematoxylin & Eosin (H&E) staining (A) reveals vascular inflammation as indicated by dilated blood vessels and inflammatory infiltrates in the hypothalamus adjacent to the third ventricle in Paigen fed ApoE/−/− mice. Focal lipid deposition as identified by Oil red O staining is observed in the vicinity of perivascular CD45-positive leucocytes (B). This is associated with an increase in the number of activated, Iba1-positive microglia (C) recruitment of CD45+ cells (D, red) and focally upregulated VCAM immunostaining (D, green). VCAM expression is also seen in the ipsilateral wall of the third ventricle but not in the contralateral part. Parallel brain sections from a representative brain are shown. *P < 0.05. Scale bars: 100 and 12.5 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)
encephalomyelitis highlights a key role of interleukin-17-producing T helper cells in recruiting immune cells into the choroid plexus (Reboldi et al., 2009). It is intriguing to speculate, therefore, that our data also highlight the possibility that such a process may occur as a result of chronic systemic inflammation alone. Alternatively, the brain inflammation may be driven by metabolic disturbances alone without the need for systemic inflammation.

Some ApoE<sup>−/−</sup> mice fed a Paigen diet also displayed brain peri-vascular areas with focal lipid deposition and with microglial and vascular inflammation, similar to that seen in large peripheral blood vessels in these mice (Stoll and Bendszus, 2006; Zadelaar et al., 2007). Our data cannot confirm whether lipid deposition in the brain is a trigger of focal inflammatory changes or only a consequence of an ongoing inflammatory response. Nevertheless, we show that atherogenic diet is associated with focal inflammatory changes in the brain of animals that develop systemic vascular disease.

In summary, we demonstrate that chronic systemic inflammatory diseases, which are primary risk factors for stroke, are associated with inflammatory changes in the brain of rodents and humans. Our data support the existence of a causal relationship between systemic inflammation and brain inflammation that may contribute to stroke and other neurological disorders. An augmented inflammatory environment in the brain of stroke-prone individuals could aggravate post-ischaemic damage if stroke occurs and further studies will address this issue. Our translational approach has shown that appropriate co-morbid animal models exist that replicate important aspects of the stroke-prone state in humans, and that these co-morbid models could help facilitate translation from experimental studies to the clinic by providing a more realistic pre-clinical setting for testing novel therapies.

**Conflict of interest**

Prof. N. Rothwell is a non-executive director of AstraZeneca, but there was no involvement of the company in any of these studies.

**Acknowledgments**

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**Appendix A. Supplementary data**


**References**

Supplementary figure. $^{[18}F]DPA-714$ uptake in the kidneys, lungs (top graph) and heart (myocardium and ventricles; bottom graph) of lean and corpulent (Cp) rats at 9, 12 and 15 months of age. There were no significant difference between lean and corpulent animals nor between ages within each strain (p>0.05, Mann-Whitney test). Data are expressed as and mean ± SD.
Interleukin-1 mediates cerebrovascular pathology induced by atherosclerosis

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Key words: Interleukin-1, atherosclerosis, cerebrovascular inflammation, leukocyte, microglia
**Abstract**

Systemic inflammatory processes contribute to brain pathology in cerebrovascular disease through mechanisms which are poorly understood. Here we show that atherosclerosis, a major systemic inflammatory disease, is associated with severe cerebrovascular inflammation in mice, and that this effect is mediated by the pro-inflammatory cytokine interleukin-1 (IL-1). Apolipoprotein E deficient (ApoE⁺/⁻) mice fed Paigen or Western diet develop vascular inflammation, microglial activation and leukocyte recruitment in the brain, which are absent in ApoE⁺/⁻ mice crossed with IL-1 type 1 receptor deficient mice (IL-R1⁺/⁻) mice. Systemic neutralisation of IL-1β with an anti-IL-1β antibody reversed aortic plaque formation and reduced inflammatory cytokine expression in peripheral organs. In the brain, vascular inflammation and leukocyte infiltration into the choroid plexus were reversed by IL-1β blockade in animals fed a Paigen diet.

These results indicate that IL-1 is a key driver of systemically-mediated cerebrovascular inflammation and that interventions against IL-1β could be therapeutically useful in atherosclerosis, dementia or stroke.
Introduction

Inflammation is a key driver in cardiovascular and cerebrovascular disease, including atherosclerosis, dementia and stroke. These diseases share common risk factors that are associated with inflammation—obesity, hypertension and diabetes (Dandona et al., 2004; Savoia and Schiffrin, 2006). In patients, there is a clear association of cardiovascular risk factors or carotid atherosclerosis with dementia, progression of Alzheimer’s disease or depression (Altman and Rutledge, 2010; Dolan et al., 2010; Silvestrini et al., 2011). Animal studies demonstrate that peripheral inflammatory conditions are associated with increased neuroinflammatory responses in the brain, and enhanced damage in response to experimentally induced stroke (Laskowitz et al., 1997; Terao et al., 2008; McColl et al., 2010; Drake et al., 2011). IL-1 is a key contributor to a diverse range of diseases, demonstrated primarily by the beneficial effects of blocking endogenous IL-1 in experimental models, most commonly with the IL-1 receptor antagonist (IL-1Ra) (Dinarello, 2011). There is evidence that IL-1 contributes to peripherally-induced neuroinflammatory diseases. Mice which lack IL-1R1, when subjected to experimental autoimmune encephalomyelitis, show a significant reduction in neuroinflammation—seen from a reduction in vascular adhesion molecule-1 (VCAM-1) and infiltrating immune cells (Li et al., 2011).

IL-1 expression is increased in human coronary arteries affected by atherosclerosis (Galea et al., 1996; Dewberry et al., 2000) and is also associated with arterial inflammation, oxidative stress, and increased blood pressure (Chamberlain et al., 2009).

In ApoE<sup>−/−</sup> mice fed high fat diets, atheroma development is related to the composition of the diet; a high fat, high cholate (Paigen) diet produces the largest effect (Chamberlain et al., 2009). Genetic deletion of IL-1R1 in ApoE<sup>−/−</sup>, Paigen diet-fed mice (ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup>) markedly reduces inflammatory responses in the periphery (Chamberlain et al., 2009). Similar results were seen in Paigen diet fed ApoE<sup>−/−</sup> mice treated with a neutralizing antibody to IL-1β (Bhaskar et al., 2011). These findings suggest that IL-1 mediates the effects of high fat diets on peripheral vascular pathology.

Our recent studies show increased cerebrovascular and brain inflammation in ApoE<sup>−/−</sup> mice fed an atherogenic high fat diet, though the mechanisms of this response are not known (Drake et al., 2011). The primary objective of this study was to test the hypothesis that the neuroinflammatory responses to atherosclerosis, induced by high fat feeding in susceptible
(ApoE<sup>−/−</sup>) mice is mediated by IL-1. To address this we determined the neuroinflammatory response to high fat feeding in ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup> mice, and the effect of immunoneutralisation of IL-1β on peripheral and central pathology in atherogenic mice.

Our data indicate that IL-1 does mediate the brain responses to atherosclerosis in mice and that IL-1β blockade provides a potential therapeutic opportunity to limit atherosclerosis, associated neuroinflammation and hence cerebrovascular disease such as stroke and vascular dementia.

**Methods**

*Animals:* Experiments were carried out in ApoE<sup>−/−</sup> (JAX 2052, Jackson Laboratories, USA) and ApoE<sup>−/−</sup>/IL-R1<sup>−/−</sup> mice, the latter generated as described previously (Chamberlain et al 2009). Both strains were bred in-house at the University of Sheffield, UK, were allowed free access to food and water, and were maintained under temperature, humidity and light-controlled conditions. All animal procedures adhered to the UK Animals (Scientific Procedures) Act (1986).

Mice aged 10 weeks were fed either a control (4.3% fat, 0.02% cholesterol), Paigen (18.5% fat, 0.9% cholesterol, 0.5% cholate, 0.26% sodium) or Western (21% fat, 0.15% cholesterol, 0.03% cholate, 0.296% sodium) diet for a period of 8 weeks, as described previously (Chamberlain et al., 2009). Body weight was recorded weekly and, as a measure of well-being and overall fitness, voluntary wheel running activity was assessed. Blood pressures were recorded weekly using a Visitech tail cuff system, as previously described (Chamberlain et al., 2009). At the end of the experimental period blood was taken from the heart, prior to transcardial perfusion. Brain, liver and spleen samples were taken and prepared as described previously (Chapman 2009; Drake et al 2011) and the aortae fixed and harvested for en face Oil Red O staining and aortic root analysis (Chamberlain et al., 2009).

*Treatment:* Mice were dosed with an anti-mouse anti-IL-1β or anti-cyclosporin A (CSA) isotype control antibody (Osborn et al., 2008, Geiger et al., 1993) according to weight (10mg/kg *i.p.*).

*Cytokine measurements:* Eleven cytokines were measured in liver homogenates and spleen homogenates: tumour necrosis factor-alpha (TNF-α), RANTES (CCL5), monocyte
chemoattractant protein-1 (CCL2), KC (CXCL1), interleukin-6 (IL-6), IL-1β, IL-1α, interleukin-17 alpha (IL-17α), interferon-gamma (IFN-γ), granulocyte-colony stimulating factor (G-CSF) and interleukin-10 (IL-10) using appropriate cytometric bead array (CBA) Flex Sets (BD Biosciences, UK) according to the manufacturers protocol. Protein concentrations were calculated using BCA assay (Pierce/Thermo Fisher Scientific, UK).

Assessment of vascular and microglial activation by immunohistochemistry: Immunohistochemistry for vascular (VCAM) and microglial (Iba1) activation was performed on free-floating brain sections. Endogenous peroxidase activity and blocking treatment were performed as stated previously. Primary antibody incubation was performed overnight using goat anti-mouse VCAM-1 1:250 (R & D Systems, UK) or rabbit anti-Iba1 1:1000 (Wako Chemicals, Germany). Sections were then incubated in appropriate biotinylated secondary antibody for 1 h (rabbit anti-goat 1:1000 and goat anti-rabbit 1:750, Vector Laboratories, UK), followed by Vectastain ABC solution (Vector laboratories, UK) before visualisation with nickel enhanced dianaminobenzidine (50 mg/ml) (Vector laboratories, UK). Sections were mounted onto gelatin coated slides, dehydrated and coverslips were applied using Depex (Fisher, UK). Images were collected on an Axiocam colour CCD camera (Zeiss, Germany) upright microscope using 10x and 20x objectives and captured using a Coolsnap ES camera (Photometrics, country) through Axiosvision software (Zeiss, Germany).

Assessment of leukocyte recruitment in the brain: Double immunofluorescence for leukocytes (CD45) and vascular activation (VCAM) was performed on free-floating brain sections. After blocking in 2% normal donkey serum (Vector Laboratories, Burlingame, CA) sections were incubated overnight at 4°C with primary antibodies: rat anti-mouse CD45 1:200 (Serotec, UK) and goat anti-mouse VCAM-1 1:250 (R & D Systems). The antigens were visualised with the adequate fluorochrome-conjugated (Alexa 594 1:750 or Alexa 488 1:500, Molecular Probes, Eugene, USA) secondary donkey antisera for two hours at room temperature. Sections were mounted onto gelatin-coated slides and coverslipped with Vectashield mounting medium containing diamidinophenylindole (Vector Laboratories, Burlingame, CA).

Images were collected on an Olympus BX51 upright microscope and captured using a Coolsnap ES camera (Photometrics, UK) through MetaVue Software (Molecular Devices, UK).
Quantitative Analysis: All analyses were performed blinded. VCAM positive blood vessels were counted in three random fields of view for each section (typically 8-10) containing rostro-caudal cerebral cortex. A score for the whole brain was obtained by averaging individual counts, and expressed as positive blood vessels per mm$^2$. Activated microglia were identified as showing; (1) increased Iba1 immunopositivity, (2) enlarged and/or amoeboid cell body, (3) complete or partial loss of thin, elongated processes. Round shaped, small Iba1-positive cells with leukocyte morphology were not counted. Activated microglia were counted in the left and right striatum on each 20µm section. Microglial counts were then totalled, averaged and expressed as activated microglia in the striatum per section hemisphere. Activated microglia also upregulate CD45 and activated Iba-1 positive microglia were found which showed CD45 positive labelling as well. Fluorescently labelled CD45 positive cells were counted in the caudal choroid plexus (-1.82 mm from Bregma) and the lateral ventricle (-1.58 mm from Bregma). The choroid plexus and ventricular ependyma were visualised by using VCAM immunofluorescence. Data on the control mice used to evaluate CD45, VCAM and Iba1 staining in ApoE$^{+/+}$/IL-1$^{−/−}$ have been published previously (Drake et al., 2011).

Statistical analysis: Comparisons between two experimental groups used an unpaired t test. Data from four groups (ApoE$^{+/+}$/ILR1$^{−/−}$ experiment) were analysed by one-way ANOVA, followed by Bonferroni’s multiple comparison post-test. Two-way ANOVA was used to determine the overall effect of diet and IL-1β on the levels of vascular and microglial activation. Blood pressure measurements were analysed by global non-linear regression, followed by an F-test. Data are presented as mean ± SD. A probability of less than 5% was regarded as statistically significant.

Results

Neuroinflammation in atherosclerotic ApoE$^{−/−}$ mice is abolished in ApoE$^{−/−}$/IL-R1$^{−/−}$ mice

In animals in which the IL-1 receptor was deleted, neuroinflammatory responses to diet feeding were abolished. More specifically, in ApoE$^{−/−}$/IL-R1$^{−/−}$ mice fed a Paigen diet microglial activation was significantly reduced compared to ApoE$^{−/−}$ mice fed a Paigen diet, and was similar to that of mice fed a control diet (Figure 1A & 1D). VCAM-1 was upregulated throughout the brain in ApoE$^{−/−}$ mice fed a Paigen diet, while there was little or no evidence of vascular activation in ApoE$^{−/−}$/IL-R1$^{−/−}$ mice fed a Paigen diet, and in these animals vascular activation was comparable to that of mice fed a control diet, and
significantly lower than in ApoE<sup>−/−</sup> mice fed a Paigen diet (Figure 1B & 1E). The enhanced leukocyte accumulation in ApoE<sup>−/−</sup> mice fed a Paigen diet was reversed in ApoE<sup>−/−</sup>/IL-R1<sup>−/−</sup> mice to levels comparable to those in mice fed a control diet (Figure 1C & 1F). In ApoE<sup>−/−</sup> mice fed a Paigen diet, activated microglia were seen adjacent to and surrounding VCAM-1 positive blood vessels. The marked decline in voluntary physical activity in ApoE<sup>−/−</sup> mice was also reversed by genetic deletion of the IL-1 receptor in the ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup> mice (Figure 1G).
Figure 1. Vascular activation, microglial activation and leukocyte accumulation are reduced in ApoE<sup>−/−</sup>/IL-1R<sup>1−/−</sup> mice fed a Paigen diet. Vascular activation was assessed through the immunostaining of the adhesion molecule VCAM. Atherosclerotic ApoE<sup>−/−</sup> mice show increased vascular activation which is significantly reduced in ApoE<sup>−/−</sup>/IL-1R<sup>1−/−</sup> mice fed a Paigen diet (A). Activated microglia as identified by increased Iba1 immunopositivity, thickened processes and irregular cell bodies was seen in atherosclerotic ApoE<sup>−/−</sup> mice fed a Paigen diet. Microglial activation was significantly reduced, to control levels, in ApoE<sup>−/−</sup>/IL-1R<sup>1−/−</sup> mice fed a Paigen diet (B). Leukocyte accumulation as shown by CD45 immunostaining was increased in atherosclerotic ApoE<sup>−/−</sup> mice fed a Paigen diet. Leukocyte accumulation was significantly reduced, to control levels, in ApoE<sup>−/−</sup>/IL-1R<sup>1−/−</sup> mice fed a Paigen diet (C). Quantification of IBA1-positive microglia (D). Quantification of VCAM-positive blood vessels (E). Quantification of CD45-positive leukocytes (F). Activated microglial cells adjacent to and surrounding VCAM-positive blood vessels in the striatum (G). Voluntary wheel running actogram for ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>/IL-1R<sup>1−/−</sup> mice, (n=2-3) (H). Error bars represent standard deviation, *p<0.05. Scale bars A) B) C) 200µm and 10µm.
Neutralisation of IL-1β reduces atherosclerotic lesion size in ApoE\(^{-/-}\) mice fed a high fat diet

Peripheral administration of an anti-IL-1β antibody successfully neutralised IL-1β (89% reduction in detectable IL-1 concentrations) in all organs where IL-1β expression was detectable regardless of diet (Figure 2).

![Figure 2. Neutralisation of IL-1β in anti-IL-1β antibody treated mice. Cytokines were measured using CBA. IL-1β was neutralised in the liver and spleen of ApoE\(^{-/-}\) mice fed a Paigen diet and in the spleen of mice fed a Western diet compared with mice treated with control antibody. Error bars represent standard deviation, *p<0.05 and **p<0.01 vs ApoE\(^{-/-}\) mice treated with a control antibody.](image)

Lesion coverage in the descending aortae was reduced significantly in IL-1β antibody treated mice, fed either a Paigen or Western diet, compared to control mice (by 34% and 45% respectively; Figure 3A & 3B). Mice treated with IL-1β antibody had a significantly smaller lesion area after feeding the Paigen diet, compared to control-antibody treated mice (Figure 3D). There was no diet-dependent difference in lesion size or percentage coverage in either the IL-1β or control antibody treatment groups. Treatment with IL-1β antibody had no statistically significant effect on systemic blood pressure in mice fed either diet (data not shown).

IL-1 neutralisation reversed the weight loss in mice fed a Paigen diet over the course of the study period (P<0.01), while there was no difference in the body weight of mice fed a Western diet, with or without intervention (Figure 1I & 3J).
Figure 3. Atherosclerotic burden is reduced after treatment with an IL-1β antibody. Atherosclerosis was assessed by Oil red O staining of whole aortae and histology of lesions at aortic roots. Inset (A-D): representative histology from Paigen diet fed mice. Whole aortae A (control antibody), B (IL-1β antibody); aortic root C (control antibody), D (IL-1β antibody). Reduction in % coverage of lesions in aortae from ApoE−/− mice fed a Western (E) and Paigen diet (F) +/− antibody treatment (both* p<0.05). Aortic root lesion size in mice fed a Western (G) and Paigen (H) diet (* p<0.05) when treated with an IL-1β antibody. Body weight changes in mice fed a Western (I) or Paigen (J) diet over the course of the study.
**IL-1β neutralisation reduces peripheral inflammation**

Consistent with the markedly reduced IL-1β levels described above, IL-1α was also reduced significantly (37%) in the liver of mice fed the Paigen diet, and in the spleen of mice fed the Western diet spleen (42%; Figure 4) but not in the spleen of mice fed a Paigen diet or the Liver of mice fed a Western diet (Supplementary figure 1). IL-1β neutralisation reduced hepatic concentrations of CCL2 (33%) in mice fed a Paigen diet, but not in mice fed a Western diet (Supplementary figure 1), and IL-6 (80%) and CXCL1 (79%) levels in spleens from mice fed a Western diet (Figure 4) but not in mice fed a Paigen diet (Supplementary figure 1).

**Figure 4.** IL-1β neutralisation has secondary anti-inflammatory effects in peripheral organs. CBA was used to measure the cytokine and chemokine levels in homogenise livers and spleen samples. IL-1α was significantly reduced in liver of ApoE<sup>−/−</sup> mice fed a Paigen diet and the spleen of mice fed a Western diet. The chemokine CCL2 was reduced in the liver of ApoE<sup>−/−</sup> mice fed a Paigen diet. The concentrations of IL-6 and CXCL1 were significantly reduced in response to IL-1β neutralisation in mice fed a Western diet. Error bars represent standard deviation, *p<0.05, **p<0.01 and ***p<0.001 vs ApoE<sup>−/−</sup> mice treated with a control antibody.
**IL-1β neutralisation attenuates vascular activation in ApoE<sup>−/−</sup> mice fed a Western diet**

Vascular and microglial activation have been reported previously in the brains of atherosclerotic ApoE<sup>−/−</sup> mice fed a Paigen diet, but no information is available on mice fed a Western diet. Mice fed the less aggressive Western diet had 57% less vascular activation when compared to mice fed a Paigen diet, indicated by a lower number of VCAM positive blood vessels (Figure 5B & 5C). Mice fed a Paigen diet showed microglial activation which was 41% higher than that of mice fed a Western diet (Figure 6). IL-1β neutralisation had no effect on vascular activation in ApoE<sup>−/−</sup> mice fed a Paigen diet, or on the number of activated microglial cells in mice fed a Paigen or western diet (Figure 6). In contrast, the neutralisation of IL-1β significantly reduced (24%) vascular activation in mice fed a Western diet (Figure 5B & 5C).

**Figure 5.** IL-1β neutralisation reduces vascular activation in ApoE<sup>−/−</sup> mice fed a western diet. Levels of vascular activation were assessed using immunostaining for the adhesion molecule VCAM. Vascular activation was assessed throughout the cerebral cortex, locations of images shown are indicated in the schematic diagram. Vascular activation was significantly reduced in ApoE<sup>−/−</sup> mice fed a western diet compared to those fed a Paigen diet. IL-1β neutralisation caused a significant reduction in vascular activation in ApoE<sup>−/−</sup> mice fed a western diet. Error bars indicate standard deviation, * p<0.05 vs ApoE<sup>−/−</sup> fed a western diet Scale bars 200µm and 50µm.
Levels of microglial activation differed between ApoE<sup>−/−</sup> mice fed a Paigen or Western diet. Levels of microglial activation were assessed through the immunostaining of IBA1. Microglial activation was assessed throughout the striatum and the location of images shown is indicated in the schematic diagram. Levels of microglial activation differed for each diet but IL-1β neutralisation had no effect. Error bars indicate standard deviation, scale bars 200µm and 10µm.

**Figure 6.** Levels of microglial activation differed between ApoE<sup>−/−</sup> mice fed a Paigen or Western diet. Levels of microglial activation were assessed through the immunostaining of IBA1. Microglial activation was assessed throughout the striatum and the location of images shown is indicated in the schematic diagram. Levels of microglial activation differed for each diet but IL-1β neutralisation had no effect. Error bars indicate standard deviation, scale bars 200µm and 10µm.

**IL-1β neutralisation attenuates the CD45+ leukocyte accumulation in ApoE<sup>−/−</sup> mice fed a Paigen diet**

Diet-dependent leukocyte accumulation was assessed in coronal brain sections and was found to occur in two different regions of the lateral ventricle (Figure 7A & D). Mice fed a Paigen diet showed increased leukocyte accumulation, principally in an area of the dorsal lateral ventricle between the hippocampus and the thalamus, as previously shown (Drake et al., 2011). Treatment with the anti-IL-1β antibody significantly attenuated the number of leukocytes accumulating in the dorsal lateral ventricle in response to the Paigen diet (Figure 7B). In contrast, mice fed a Western diet showed little accumulation of leukocytes in this region of the lateral ventricle compared to the Paigen diet (Figure 7C). The accumulation of leukocytes was found to extend ventrally to the choroid plexus of the ventricular space in mice fed a Paigen diet (Figure 7E) and mice fed a Western diet also show increased leukocyte infiltration in this ventral region, though numbers are reduced compared to the Paigen fed (Figure 7F). Overall, the anti-IL-1β antibody reduced leukocyte accumulation in the ventral ventricle compared to control antibody (P<0.05, Two way ANOVA).
Leukocytes accumulate in the choroid plexus of the lateral ventricle in ApoE<sup>p/p</sup> mice fed a Paigen diet and, to a lesser extent, a Western diet, which is attenuated in the Paigen diet by an anti-IL-1β antibody treatment. Fewer CD45<sup>p</sup> positive cells accumulate in this region in mice fed a western diet. Accumulation of leukocytes in this region is not seen in mice fed a Western diet. Fewer CD45-positive cells accumulate in this region in mice fed a western diet however IL-1β antibody treatment has no effect. Quantification of the total number of CD45-positive leukocytes in the regions shown in A and B in mice fed a Western (C) or Paigen diet (D). Error bars represent standard deviation, *p<0.05 vs ApoE<sup>p/p</sup> mice treated with a control antibody.

**Discussion**

We show that CNS pathology is associated with the development of atherosclerosis in high fat fed ApoE<sup>p/p</sup> mice and, through genetic deletion of IL-1R1 or neutralisation of IL-1β, provide evidence that this is mediated by IL-1. These findings identify IL-1 as a key driver in the development of cerebrovascular inflammation in response to atherogenic diet and systemic vascular disease.
Neutralisation of IL-1β had anti-inflammatory effects in peripheral organs. High fat diet feeding causes a raised level of hepatic inflammation due to an increased accumulation of cholesterol (Tous et al., 2005). Treatment of hepatic cells with IL-1β also causes a raised level of cholesterol uptake and accumulation (Ma et al., 2008). Therefore the reduced hepatic inflammation seen in this study could be due to the decreased availability of IL-1β to the hepatic cells and thus a decreased uptake of cholesterol. Recent data also implicates central IL-1 signalling in muscle atrophy which was found to be dependent on the hypothalamic-pituitary-adrenal axis (Braun et al., 2011).

Treatment with an anti-IL-1β antibody attenuated brain leukocyte accumulation in the Paigen fed animals, but no effect was seen in mice fed a less aggressive Western diet (Vergnes et al., 2003), the latter showing a much lower brain leukocyte invasion compared to Paigen fed animals. Vascular activation was reduced by neutralisation of IL-1β in mice fed a Western diet, but no effect was seen in Paigen diet fed animals, and no effect was seen in either diet in relation to microglial activation. These observations are not consistent with the findings in ApoE<sup>p/p</sup>/IL-R1<sup>p/p</sup> mice fed a Paigen diet and may be because of limited brain penetration of the anti-IL-1β antibody, or because IL-1α, which can also mediate activation of IL-1R1, contributes to changes in the brain and cerebrovasculature.

ApoE<sup>−/−</sup> mice fed either a Western or Paigen diet are known to develop atheroma throughout the aorta (Getz and Reardon, 2006). Treatment with an anti-IL-1β antibody caused a significant reduction in the atherosclerotic lesion area in the descending aorta in mice fed both a Paigen and Western diet and in the aortic root in mice fed a Paigen diet. These data are consistent with a recent study, which reported similar effectiveness of another anti-IL-1β antibody treatment for atherosclerosis (Bhaskar et al., 2011). Since ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup> mice fed an atherosclerotic diet also develop reduced peripheral pathology (Chamberlain et al., 2009) it is not possible from the findings of this study to determine the specific site of IL-1 action in driving CNS pathology. The reduced impact of the high fat diet in mice lacking the IL-1 receptor may be due to the absence of IL-1 signalling within the cerebrovasculature or in the brain, or secondary to the amelioration of peripheral pathology.

Though CNS inflammation is observed in response to peripheral disease it may itself contribute to the actual development of vascular diseases, recent experimental data indicating a role for centrally-mediated signalling by inflammatory cytokines such as IL-1β and TNFα in neurogenic hypertension or metabolic disease (Ye et al., 2000; Shi et al.,
Thus, interventions against central and systemic effects of vascular diseases could have therapeutic potential. Elevated systemic inflammatory burden is considered a high risk for cardio- and cerebrovascular diseases (Spagnoli et al., 2007; McColl et al., 2009; Denes et al., 2010). There is clear association between depression and cardiovascular/metabolic disease (Plante, 2005; Williams and Steptoe, 2007; Somberg and Arora, 2008) and IL-1 signalling has been linked closely with the development of decreased motivational disorders such as depression (Koo and Duman, 2009; Diniz et al., 2010; Lehto et al., 2010).

Therefore, patients with multiple risk factors for heart disease or stroke might benefit from neutralisation of systemic IL-1β activity. Similarly, the potential effects of high fat diet and atherosclerosis on cognitive decline and depression might be reduced by interventions against IL-1 signalling.

**Conclusions**

We present evidence that the cytokine IL-1 contributes to cerebrovascular pathology in atherosclerotic mice, and that a clinically relevant treatment attenuates the peripheral and brain responses to high fat diet.

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**Conflict of Interest**

NR is a nonexecutive director of AstraZenca, but this has no relation to the current research. HG is an employee of Novartis Pharma AG.
References


Supplementary figure 1. Reduced cytokines levels following IL-1β neutralisation was not seen in all organs. CBA was used to measure the cytokine and chemokine levels in homogenise livers and spleen samples. IL-1α, IL-1β and CCL2 were not reduced in the livers of ApoE^-/- mice fed a Western diet. IL-1α, IL-6 and CXCL1 were not reduced in the spleen of ApoE^-/- mice fed a Paigen diet.
Systemic and brain inflammation in mice in response to combined risk factors for stroke

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Systemic and brain inflammation in mice in response to combined risk factors for stroke

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Abstract

Atherosclerosis and respiratory infections are major risk factors for stroke and are known to involve highly inflammatory components however it is poorly understood how a combination of both risk factors affects stroke risk. Here we report that infection of the ApoE<sup>p/p</sup> mouse, fed a Paigen diet to induce atherosclerosis, with <i>S. Pneumoniae</i>, the primary cause of pneumonia, does not induce spontaneous stroke. We did however observe raised levels of vascular activation and an alteration in leukocyte accumulation in infected mice. T cell, B cell and macrophage level changes to both diet and infection were found to differ between ApoE<sup>p/p</sup> mice and control, C57BL/6, mice. Levels of the proinflammatory cytokines IL-1, IL-6 and IL-17 were also increased in response to <i>S. Pneumoniae</i> infection in plasma, spleen and liver. These data indicate that atherosclerosis and <i>S. Pneumoniae</i> infections not only have systemic inflammatory mechanisms but also effects which extend to the brain. The neuroinflammatory aspects of these diseases should therefore be considered when modelling diseases such as stroke for which they are key risk factors.
Introduction

Stroke is a leading cause of death and disability and is estimated to cost £8 billion per year in England alone (Scharaschkin et al., 2010). Although widely studied, there are still limited treatments for stroke and this has been attributed to a number of factors, including poor experimental design and the lack of studies considering relevant co-morbidities and risk factors (Fisher et al., 2009). Risk factors such as obesity, diabetes, atherosclerosis and infection have been identified as predisposing patients to a stroke or impairing outcome (Amarenco et al., 1996; Clayton et al., 2008; Eeg-Olofsson et al., 2009), yet are very rarely considered in experimental studies.

Bacterial infections, such as pneumonia, in the weeks prior to an ischaemic event are associated with an increased risk of stroke (Hassan et al., 2006), and in experimental stroke studies the induction of infection prior to ischaemia leads to an exacerbation of the resulting lesion volume (Denes et al., 2010; Muhammad et al., 2011). Further evidence of the relationship between infection and stroke is demonstrated by the observation that vaccination, of patients, against influenza leads to a 50% reduction in stroke occurrence (Grau et al., 2005). Experimentally, administration of bacterial LPS prior to stroke causes an increased lesion volume via an MMP-9-mediated mechanism (McColl et al., 2008). Infection has also been identified as a key risk factor of stroke in young patients (Emsley and Hopkins, 2008; Lee et al., 2008), a cohort without any atherosclerotic burden, indicating that the ischaemic event occurs independently of plaque rupture.

Despite this, the rupture of atherosclerotic plaques is still the major cause of occluding clots in many ischaemic conditions, including stroke and myocardial infarction (Fuster et al., 1992; Amarenco et al., 1996). Plaque rupture occurs due to a breakdown of the fibrous cap covering a stable lesion and this is thought to occur either through physical stress or enzymatic breakdown (Spagnoli et al., 2007; Li and Gillard, 2008). Enzymes such as MMPs have been implicated in plaque breakdown and these MMPs can come from macrophages, neutrophils and invading bacteria (Heo et al., 2011). Bacterial infections have been identified as a possible contributor to the pathogenesis of atherosclerosis and plaque rupture events (Chiu, 1999), which could explain the link to stroke described above. *C. pneumoniae* has been detected in various stages of atherosclerotic plaque development (Belland et al., 2004), and *P. gingivalis*, a key bacteria in oral disease, has been shown to cause alterations in vascular reactivity and to accelerate atherosclerosis (Lalla et al., 2003; Pereira et al., 2011). *S. pneumoniae* is known to cause septic shock, which has been
associated with multi organ failure and raised systemic inflammation (Andonegui et al., 2009), which might lead towards a plaque rupture prone state, similar to that seen with other bacterial organisms. Vaccination against *S. pneumoniae* has been shown to cause a reduction in atherosclerosis and also a reduction in key markers associated with atherogenesis (Binder et al., 2003). However, the effect of *S. pneumoniae* on actual plaque rupture has not been studied.

Infections cause a rise in the inflammatory profile of patients, including increased IL-6, CRP and IL-1 expression. Patients with risk factors for stroke have also been shown to have an increased level of systemic and brain inflammation (Dandona et al., 2004; Patel et al., 2008; Drake et al., 2011). In agreement mouse models of atherosclerosis have been shown to have increased levels of vascular activation, microglial activation and leukocyte infiltration prior to an ischaemic event (Drake et al., 2011). Given the association of both infection and systemic inflammation with the incidence of stroke the primary aim of this study was to determine whether a combined challenge of atherosclerosis and infection might result in spontaneous stroke in mice. A secondary aim was to determine if a change in the previously reported brain inflammation in atherogenic mice is exacerbated in response to infection.

**Methods**

**Animals**

Experiments were carried out in male ApoE−/− mice (JAX 2052, Jackson Laboratories, USA) bred in-house at the University of Manchester and control C57BL/6 mice (Harlan, UK). Animals were allowed free access to food and water and were maintained under temperature, humidity and light-controlled conditions. All animal procedures adhered to the UK Animals (Scientific Procedures) Act (1986). ApoE−/− and C57BL/6 mice aged 12 weeks were fed either an atherogenic Paigen diet (18.5% fat, 0.9% cholesterol, 0.5% cholate, 0.26% sodium, Special Diet Services, UK) or a normal chow diet (4.3% fat, 0.02% cholesterol, Special Diet Services, UK) for 8 weeks.

**Infection**

An infection protocol was initially established to allow a low grade infection to be present for a period of 7 days. To achieve this, animals were infected on three separate days to prevent clearance of the bacterium. Initial experiments were performed using the following protocol; day 0 (4 x 10⁶ bacteria), day 2 (4 x 10⁶ bacteria) and day 5 (8 x 10⁶ bacteria).
This protocol however was found to cause animals to develop severe infections and the experiment was thus ended early. A reduction in the initial dose of bacterium was found to cause the desired infection and therefore after 8 weeks of diet the following infection protocol was used throughout; day 0 (2 x 10^6 bacteria), day 2 (4 x 10^6 bacteria) and day 5 (8 x 10^6 bacteria). To infect, mice were lightly anaesthetised with 2.5% isoflurane (in 30% O_2/ 70% N_2O) and suspended vertically to ensure the airway was unobstructed. *S. pneumoniae* (50µl) ATCC 49619 was pipetted onto the nostril to allow subsequent dissemination throughout the lungs. Animals were defined as being successfully infected due to their physical appearance, including piloerection and increased respiration rate. Mice showing reduced activity and a hunched appearance were culled immediately on appearance of these symptoms. All other infected mice were culled on day 6 after the first infection, at the same time as non-infected, control mice.

**Tissue processing**

Under terminal anaesthesia, prior to whole body transcardial perfusion, blood was taken from the heart using 3.8% sodium citrate (1:10) as an anti-coagulant. Mice were then perfused transcardially with saline at a rate of 10ml/min using a syringe perfusion pump (Harvard apparatus, UK), and the liver and spleen removed. Half the spleen was minced and transferred to FACS buffer (PBS supplemented with 0.1% bovine serum albumin (BSA) and 20mM sodium azide (NaN_3)) and the other half spleen, along with the liver, were snap frozen on dry ice. The left hind leg was removed and both the tibia and fibula were flushed with FACS buffer to obtain the bone marrow cells. PFA (4% in PBS; Sigma, UK) was then perfused at a rate of 10ml/min using a syringe perfusion pump (Harvard apparatus, UK), prior to removing the brain and post-fixing in 4% PFA at 4°C for 24 h. Brains were subsequently cryoprotected in PBS containing 20% sucrose for 24 h. Five alternate sets of 20 µm thick coronal brain sections were cut on a sledge microtome (Bright series 8000; Bright Instruments, Huntingdon, UK). All sections were collected into an antifreeze solution (containing 30% ethylene glycol (Sigma, UK) and 20% glycerol (Fisher, UK) in PBS) and stored at -20°C until processing.

**Flow cytometry**

Spleen, bone marrow and blood cells were isolated and stained with CD3 (T cells) and CD19 (SSC low - B cells), CD14, CD11b (SSC high - granulocytes), MHC class II, XCR2, CCR5 and CD11c (BD Biosciences) alone or in combination. Cells were acquired on
FACSCalibur or LSRII flow cytometers (BD Biosciences). Acquisition and analysis of data were performed using CellQuest Pro or FACS Diva software (BD Biosciences).

**Cytokine measurements with cytometric bead array (CBA)**

Liver and spleen samples were homogenised as described previously (Chapman et al., 2009) and protein concentrations calculated using a BCA assay (Pierce/Thermo Fisher Scientific). Plasma samples were diluted 1:5 with assay diluents and the following eleven cytokines were measured: tumour necrosis factor-alpha (TNF-α), RANTES (CCL5), monocyte chemoattractant protein-1 (CCL2), KC (CXCL1), interleukin-6 (IL-6), IL-1β, IL-1α, interleukin-17 alpha (IL-17α), interferon-gamma (IFN-γ), granulocyte-colony stimulating factor (G-CSF) and interleukin-10 (IL-10) using appropriate cytometric bead array (CBA) Flex Sets (BD Biosciences, UK) according to the manufacturers protocol. Protein concentrations were calculated using BCA assay (Pierce/Thermo Fisher Scientific, UK).

**Immunohistochemistry**

Immunohistochemistry was performed on 20µm free-floating brain sections. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide (Sigma) in dH2O and sections were treated with 2% normal serum (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were incubated overnight in antibody diluent (0.1 M PBS + 0.3 % Triton X-100, Sigma) using either goat anti-mouse VCAM-1 1:250 (R & D Systems, UK) or rabbit anti-Iba1 1:1000 (Wako Chemicals, Germany) antibodies. Sections were then incubated in appropriate biotinylated secondary antibody for 1 h (rabbit anti-goat 1:1000 and goat anti-rabbit 1:750, Vector Laboratories, UK), before incubation in Vectastain ABC solution (Vector laboratories, UK). Colour was developed by nickel enhanced diaminobenzidine (50 mg/ml) incubation (Vector laboratories, UK) and sections mounted onto gelatine coated slides, dehydrated and coverslipped using Depex (Fisher, UK). Images were collected on an Axiocam colour CCD camera (Zeiss, Germany) upright microscope using 10x and 20x objectives and captured using a Coolsnap ES camera (Photometrics) through Axiovision software (Zeiss, Germany).

**Immunofluorescence**

Double immunofluorescence was performed on free-floating brain sections. After blocking in 2% normal donkey serum (Vector Laboratories) sections were incubated overnight at 4°C in primary antibodies: rat anti-mouse CD45 1:200 (Serotec, UK) and goat anti-mouse VCAM-1 1:250 (R & D Systems). The antigens were visualized with the adequate
fluorochrome-conjugated (Alexa 594 1:750 or Alexa 488 1:500, Molecular Probes) secondary donkey antisera for 2 h at room temperature. Sections were mounted onto gelatin-coated slides and cover-slipped with ProLong mounting medium containing diamidinophenylindole (Invitrogen, UK).

Images were collected on an Olympus BX51 upright microscope using 4x, 10x, 40x and 60x objectives and captured using a CoolSnap ES camera (Photometrics, UK) through MetaVue Software (Molecular Devices, UK). Specific band pass filter sets for DAPI, FITC and Texas red were used to prevent bleed through from one channel to the next.

**Quantitative Analysis**

All quantitative analysis was performed under blinded conditions. VCAM positive blood vessels were counted in three random fields of view for each section (typically 8-10) containing rostro-caudal cerebral cortex. A score for the whole brain was obtained by averaging individual counts and this was expressed as positive blood vessels per mm².

Activated microglia were identified as showing; (1) increased Iba1 immunopositivity, (2) enlarged and/or amoeboid cell body, (3) complete or partial loss of thin, elongated processes. Round shaped, small Iba1-positive cells with leukocyte morphology were not counted. Activated microglia were counted in the left and right striatum on each 20µm section. Microglial counts were then totalled, averaged and expressed as activated microglia in the striatum per section hemisphere. Activated microglia also upregulate CD45 and activated Iba-1 positive microglia were found which showed CD45 positive labelling as well.

Fluorescently labelled CD45 positive cells were counted in the caudal choroid plexus (-1.82 mm from Bregma) and the lateral ventricle (-1.58 mm from Bregma). The choroid plexus and ventricular ependyma were visualised by using VCAM immunofluorescence.

**Statistical Analysis**

Data obtained from quantitative analysis of VCAM, microglia and leukocyte immunohistochemistry staining was analysed, using Statview software, by a repeated measures 3-way ANOVA with Scheffe’s post hoc analysis. CBA and flow cytometry data was analysed using individual 3-way ANOVA tests with Scheffe’s post hoc analysis. Significance was determined at the 5% level.
Results

Systemic infection does not cause spontaneous stroke but leads to an increase in vascular activation

We did not observe any evidence of an ischaemic event in the brains of mice after infection. Systemic *S. pneumoniae* infection was found to cause an overall increase in the levels of vascular activation, although post hoc analysis was unable to discriminate differences among individual groups (Figure 1).

![Figure 1](image_url)

Figure 1. Vascular activation is increased in response to *S. pneumoniae* infection. Levels of vascular activation were assessed, throughout the cerebral cortex, through the immunostaining of the adhesion molecule VCAM. (A) *S. pneumoniae* infection caused an increase in the levels of vascular activation in all groups. (B) Schematic diagram indicating the location of images in A. (C) Quantitative analysis of vascular activation with three way ANOVA comparisons (scatter plots show mean and standard deviation bars). CC – C57BLJ/6 mouse fed a chow diet, AC – ApoE<sup>−/−</sup> mouse fed a chow diet, CP – C57BLJ/6 mouse fed a Paigen diet, AP – ApoE<sup>−/−</sup> mouse fed a Paigen diet, ICC – infected C57BLJ/6 mouse fed a chow diet, IAC – infected ApoE<sup>−/−</sup> mouse fed a chow diet, ICP – infected C57BLJ/6 mouse fed a Paigen diet, IAP – infected ApoE<sup>−/−</sup> mouse fed a Paigen diet. Scale bars 200µm and 50µm (insert).
Microglial activation was assessed throughout the striatum in the brains of mice and was found to be of a similar level in all groups examined, systemic infection having no effect (Figure 2).

Figure 2. Microglial activation did not change in response to a *S. pneumoniae* infection. (A) Microglial activation did not change between non-infected animals and infection did not change the levels of microglial activation. (B) Schematic diagram indicating the location of images shown in A. (C) Quantification of microglial activation (scatter plots show mean and standard deviation bars). CC – C57BLJ/6 mouse fed a chow diet, AC – ApoE<sup>p/p</sup> mouse fed a chow diet, CP – C57BLJ/6 mouse fed a Paigen diet, AP – ApoE<sup>p/p</sup> mouse fed a Paigen diet, ICC – infected C57BLJ/6 mouse fed a chow diet, IAC – infected ApoE<sup>p/p</sup> mouse fed a chow diet, ICP – infected C57BLJ/6 mouse fed a Paigen diet, IAP – infected ApoE<sup>p/p</sup> mouse fed a Paigen diet. Scale bar 200µm.

**Systemic infection causes a decrease in leukocyte accumulation in the caudal choroid plexus**

Leukocytes were found to accumulate in the caudal choroid plexus of atherosclerotic ApoE<sup>p/p</sup> mice fed a Paigen diet (AP) and quantitative analysis found a 6-fold increase in accumulation in these mice when compared to infected or non-infected C57BLJ/6 mice fed a Paigen (ICP, CP) or chow diet (ICC, CC) and infected or non-infected ApoE<sup>p/p</sup> mice fed a chow diet (IAC, AC) (Figure 3). *S. pneumoniae* infection caused an overall decrease in the
accumulation of leukocytes in the choroid plexus, although post hoc analysis was unable to discriminate differences among individual groups (Figure 3).

Figure 3. Leukocyte accumulation in the caudal choroid plexus was decreased in response to *S. pneumoniae* infection. (A) Leukocyte accumulation was found to be increased in ApoE<sup>p/p</sup> mice fed a Paigen diet. Infection caused an overall decrease in the accumulation of leukocytes in infected groups. (B) Schematic diagram indicating the location of quantification of leukocyte accumulation and the location of images in A. (C) Quantitative analysis of leukocyte accumulation with three way ANOVA comparisons ($$P<0.001$$ vs CC, AC, CP, ICC, IAC and ICP: # $$P<0.05$$ vs CC, AC and CP: ## $$P<0.01$$ vs ICC, IAC and ICP (scatter plots show mean and standard deviation bars)). CC – C57BL/6 mouse fed a chow diet, AC – ApoE<sup>p/p</sup> mouse fed a chow diet, CP - C57BL/6 mouse fed a Paigen diet, AP – ApoE<sup>p/p</sup> mouse fed a Paigen diet, ICC – infected C57BL/6 mouse fed a chow diet, IAC – infected ApoE<sup>p/p</sup> mouse fed a chow diet, ICP – infected C57BL/6 mouse fed a Paigen diet, IAP – infected ApoE<sup>p/p</sup> mouse fed a Paigen diet. Scale bars 200µm and 10µm (insert).
Systemic infection causes a systemic inflammatory response

Changes seen in the percentages of bone marrow cells was reflected in the total number of cells however, due to an error in processing, the total number of spleen cells for each labelling group was not available. We are however confident that the total number of spleen cells is reflected in the percentages, as seen in other experiments. A summary of the changes in the percentage of B cell, T cell and granulocyte cells following systemic infection in all groups in blood, bone marrow and spleen can be found in Table 1.

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| Table 1. Summary table of cellular changes in response to systemic inflammation. Individual group numbers of B cells (CD19+ SSC low), T cells (CD3+) and granulocytes (CD11b+ SSC high) in the blood, bone marrow and spleen. Arrows indicate increases or decreases in the number of cells in individual groups and a dash indicates no change in the levels. CC – C57BLJ/6 mouse fed a chow diet, AC – ApoE<sup>−/−</sup> mouse fed a chow diet, CP – C57BLJ/6 mouse fed a Paigen diet, AP – ApoE<sup>−/−</sup> mouse fed a Paigen diet, ICC – infected C57BLJ/6 mouse fed a chow diet, IAC – infected ApoE<sup>−/−</sup> mouse fed a Paigen diet, ICP – infected C57BLJ/6 mouse fed a Paigen diet, IAP – infected ApoE<sup>−/−</sup> mouse fed a Paigen diet.

The percentage of CD19+ SSC low B cells in the blood were found to be strongly dependent on genotype and diet (Figure 4Ai). CP mice showed an increased level (1.3 to 5-fold) of B cells in the blood compared to all other groups, and this was not affected by infection (Figure 4Ai). ApoE<sup>−/−</sup> mice had 3 fold less (P<0.001) T cells in the blood compared to C57BLJ/6 mice (P<0.001). Feeding of a Paigen diet increased the percentage of T cells in the blood only in C57BLJ/6 mice (Three way ANOVA P<0.001), while infection did not alter the percentage of T cells in any group (Figure 4Aii).

In the bone marrow, feeding of a Paigen diet caused an overall reduction (P<0.001) in the percentage of CD19+ SSC low B cells, though differences between individual groups could not be resolved (Figure 4Bii). Feeding of a Paigen diet caused a decrease in the
percentage of T cells in the bone marrow in both C57BL/6 and ApoE<sup>−/−</sup> mice (Figure 4Biii). The percentage of T cells in the bone marrow were increased in all groups following infection (three-way ANOVA P<0.001) with a 2 fold increase seen in ICC mice. AP and AC mice showed a raised percentage of CD11b<sup>+</sup> cells in the bone marrow prior to infection. Following infection, the percentage of CD11b<sup>+</sup> cells in the bone marrow was significantly reduced by 15%. Infection caused a decrease in the percentage of CD11b<sup>+</sup> SSC high granulocytes in the bone marrow (Three way ANOVA P<0.001) (Figure 4Bi and iv).

The percentage of CD19<sup>+</sup> SSC low B cells in the spleen were dependent on genotype (three way ANOVA P<0.001), and infection had no effect, post hoc analysis revealing no differences between any groups (Figure 4Ci). In the spleen, CD3<sup>+</sup> T cell percentages were dependent on genotype and infection (three-way ANOVA P<0.001). T cells in infected ApoE<sup>−/−</sup> mice were one and half times greater than infected C57BL/6 mice and all non-infected groups (Figure 4Cii). In the spleen raised numbers of CD11b<sup>+</sup> SSC high granulocytes were seen in C57 mice prior to infection. Infection caused an overall decrease in the percentage of cells (Figure 4Ciii, Three way ANOVA P<0.001) Three-way ANOVA was performed for all groups and a summary of the findings can be found in table 3).
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Table 2. Summary of 3-way ANOVA results. 3 way ANOVA results for B cells (CD19+ SSC low), T cells (CD3+) and granulocytes (CD11b+ SSC high) in the blood, bone marrow and spleen * P<0.05, ** P<0.01 and *** P<0.001. CC – C57BL/6 mouse fed a chow diet, AC – ApoE<sup>−/−</sup> mouse fed a chow diet, CP - C57BL/6 mouse fed a Paigen diet, AP – ApoE<sup>−/−</sup> mouse fed a Paigen diet, ICC – infected C57BL/6 mouse fed a chow diet, IAC – infected ApoE<sup>−/−</sup> mouse fed a chow diet, ICP – infected C57BL/6 mouse fed a Paigen diet, IAP – infected ApoE<sup>−/−</sup> mouse fed a Paigen diet.
Figure 4. Number of cells in response to diet and *S. pneumoniae* infection vary between ApoE<sup>p/p</sup> and C57BLJ/6 mice. (A) Total number of B cells (CD19<sup>+</sup> SSC low) i) and T cells (CD3<sup>+</sup>) ii) in the blood. A Paigen diet caused an increase in the number of B cells (CD19<sup>+</sup> SSC low) ($P<0.05$ vs CC; $$$P<0.001$ vs IAC, AC, IAP and AP; # $P<0.05$ vs CC; ## $P<0.01$ vs IAP and AP) and T cells (CD3<sup>+</sup>) ($P<0.05$ vs AC; $$$$P<0.001$ vs IAP and AP; # $P<0.05$ vs AP; ## $P<0.01$ vs IAP) in the blood in C57BLJ/6 mice. Infection had no effect on the number of T and B cells in the blood. (B) Example dot plots for granulocytes (CD11b<sup>+</sup> SSC high (large right hand gate)) in the bone marrow i). B cells (CD19<sup>+</sup> SSC low) in the bone marrow ii) were reduced in mice fed a Paigen diet however no effect of infection was seen ($P<0.05$ vs IAP and CP). Total number of T cells (CD3<sup>+</sup>) in the bone marrow increased in response to infection iii) ($P>0.05$ vs CC and ICP). ApoE<sup>p/p</sup> mice showed an increased number of granulocytes (CD11b<sup>+</sup> SSC high) in the bone marrow iv) ($$$P<0.01$ vs IAC and ICC, $$$$P<0.001$ vs AC, CC, ICP and IAC). Percentage populations of B cells (CD19<sup>+</sup> SSC low) in the bone marrow v) were reduced in mice fed a Paigen diet however no effect of infection was seen. Percentage populations of T cells (CD3<sup>+</sup>) in the bone marrow vi) increased in response to infection. ApoE<sup>p/p</sup> mice showed an increased percentage of granulocytes (CD11b<sup>+</sup> SSC high) in the bone marrow vii) ($P<0.05$ vs ICC and ICP; ## $P<0.01$ vs IAC, IAP and CC ### $P<0.001$ vs ICC and ICP). C) Percentage population of B cells (CD19<sup>+</sup> SSC low) in the spleen ii) were found to be dependent on genotype. Percentage population of T cells (CD3<sup>+</sup>) in the spleen ii) were increased in IAC mice compared with ICC mice ($P<0.05$). In the spleen granulocytes (CD11b<sup>+</sup> SSC high) were increased in C57BLJ/6 mice prior to infection ($$$$P<0.001$ vs IAC, AC, IAP, AP, ICC and ICP, ## $P<0.01$ vs IAC and IAP). Levels were found
to be dependent on genotype and decreased following infection. Scatter plots show mean and standard deviation.

**Cytokine levels are altered in response to systemic infection**

In the spleen an overall increase in the levels of IL-6 were seen in response to infection (three-way ANOVA P<0.05) (Figure 5Aiii). The variability in levels of IL-6 within groups of infected animals (Coefficient of variation 45.67%-106.75%) was greater than that of groups of non-infected animals (Coefficient of variation 0.93%-26.26%) and may account for the lack of significance in individual groups. Infection caused an overall increase in levels of IL-1α in the spleen samples (P<0.05) (Figure 5Biii). IL-1α levels were dependent on both genotype and diet in the spleen (Three-way ANOVA P<0.001 and P<0.05) and the plasma (Three-way ANOVA both P<0.001). Levels of IL-1α were increased in AP and IAP animals in both the liver (7 fold) and the spleen (3 fold). Infection caused an increase in the levels of IL-17 in the plasma in all groups (Figure 5).
Figure 5. *S. pneumoniae* infection causes a raised level of IL-6 in the liver (A). IL-1α was raised in the liver in AP and IAP mice ($$$ P<0.001 vs IAC, AC, ICC, CC, ICP and CP; # P<0.05 vs ICP; ## P<0.01 vs CP; ### P<0.001 vs IAC, AC, ICC and CC) (B). In the plasma, levels of IL-1α were found to be dependent on diet and were increased following infection (C). Levels of IL-1α in the spleen (D) were increased in AP and IAP mice ($ P<0.05 vs ICC, CC and CP; # P<0.05 vs ICC, CC and CP). In plasma samples, IL-17 (E) was not detectable (n.d.) prior to infection. Following infection levels of IL-17 were increased in ICC ($$ P<0.01 vs AC, AP, CC and CP, # P<0.05 vs ICC, ### P<0.001 vs AC, AP, CC and CP, £ P<0.05 vs AC, AP, CC and CP). Summary of 3-way ANOVA results for cytokine levels as measured by CBA in the liver, spleen and plasma (F). * P<0.05, ** P<0.01 and *** P<0.001. CC – C57BLJ/6 mouse fed a chow diet, AC – ApoE⁻/⁻ mouse fed a chow diet, CP - C57BLJ/6 mouse fed a Paigen diet, AP – ApoE⁻/⁻ mouse fed a Paigen diet, ICC – infected C57BLJ/6 mouse fed a chow diet, IAC – infected ApoE⁻/⁻ mouse fed a chow diet, ICP – infected C57BLJ/6 mouse fed a Paigen diet, IAP – infected ApoE⁻/⁻ mouse fed a Paigen diet.

**Discussion**

Here we report that systemic infection with *S. pneumoniae* causes an increase in cerebrovascular activation and a decrease in leukocyte accumulation in the brain. We did not however observe any behavioural and/or histological changes which might indicate the
occurrence of spontaneous cerebral ischaemia in response to infection. Systemically, infection caused changes in the percentage of granulocytes and T cells and increased the expression of IL-6, IL-17 and IL-1α. However, the most prominent factors in modulating the immununological response was the genotype of the mice, and the diet they were fed. This data suggests that *S. pneumoniae* infection induces a systemic inflammatory response, and also promotes changes in the brain.

Spontaneous ischaemic stroke is not a common occurrence in rodents and only observed sporadically in hypertensive animals (Okamoto et al., 1974) which, may account for the lack of spontaneous strokes in this study. Mice showed both behavioural and immunological responses to infection, as shown by raised spleen IL-6 levels, increased granulocytes and T cells, to indicate successful infection. Levels of IL-6 in the spleen of infected mice were not increased significantly, in individual groups, compared to non-infected controls and thus the infection chosen may not have been serious enough to induce plaque rupture. Unstable plaques have been observed in mice following infections with *C. pneumoniae* and high levels have been detected post mortem in ruptured plaques but little is known about the stability of plaques in the presence of *S. Pneumoniae* (Higuchi et al., 2000; Ezzahiri et al., 2003).

Although no cerebral ischaemic events were observed in response to infection, we did observe neuroinflammatory changes, an overall increase in the levels of vascular activation following infection being seen. A similar activation of endothelial cells was seen in the lungs following intranasal *S. Pneumoniae* infection (Kadioglu et al., 2011). Furthermore, *in vitro* experiments have shown that endothelial cells exposed to *S. pneumoniae* have an increased production of pro-inflammatory cytokines, suggesting vascular activation (Banerjee et al., 2010). This is similar to the response of the brain endothelium to systemic diseases such as obesity and atherosclerosis, in the absence of brain pathology, (Drake et al., 2011). We also observed raised levels of IL-1α in plasma samples from infected mice. Earlier work has shown that increased expression of the proinflammatory cytokine IL-1 is associated with increased levels of vascular activation in the brain (Chapter 2), which could explain the raised levels of neuroinflammation seen in the presence of infection.

The accumulation of leukocytes in the brain is known to occur in CNS pathologies such as stroke and Alzheimer’s disease (Wyss-Coray and Mucke, 2002; Buck et al., 2008). We recently showed that leukocytes also accumulate in the brains of atherosclerotic ApoE−/− mice fed high fat (Drake et al., 2011). However, only very few reports are available on the
Effect of a systemic infection on leukocyte accumulation in the brain and we show an overall decrease in the levels of leukocyte accumulation in the brains of Paigen fed atherosclerotic mice after infection. *S. pneumoniae* involves the migration of a number of different leukocytes to the site of infection (Nakasone et al., 2007). It is likely that production/release of inflammatory cells from the bone marrow is altered in infected mice, and/or levels of chemokines may be greater in the lung at the site of infection, when compared to the brain, thus explaining the decreased number of cells. In cases of severe meningitis as a result of *S. pneumoniae* infection, leukocytes have been shown to enter into the brain and microglial cells have been activated, however this is in the presence of invading bacteria (Gerber and Nau, 2010).

*S. pneumoniae* infections induce a T cell response in the bone marrow and spleen as seen in this study (Paterson and Orihuela, 2010). Infected ApoE<sup>−/−</sup> mice also showed an increased CD8<sup>+</sup> T cell response in the spleen which, has previously been shown to occur in response to hypercholesterolemia (Kolbus et al., 2010). Although this is not seen prior to infection, these cells could be primed and therefore increase rapidly in response to infection. Raised numbers of T cells were also seen in the blood in CP mice prior to infection, as were B cells, and it appears as though the hyperlipidemic and hypercholesterolemic state induced by a Paigen diet causes an increase in the release of B and T lymphocytes from the bone marrow and spleen as seen previously (Gomes et al., 2010; Maganto-Garcia et al., 2011). These data indicate that both infection and a Paigen diet are working via a similar mechanism although induced differently according to the genotype of the mice. The identification of similar mechanism was also seen in the levels of IL-1α which rose following infection to a level similar to that of AP mice prior to infection. The levels of IL-1α however were not increased further in the IAP mice and therefore the inflammatory mechanisms seen appear to be overlapping and not additive. This could further explain the lack of cerebral ischaemia events, as we hypothesise that the inflammatory burden needs to be driven to towards a plaque rupture prone state and this may not have achieved in these experiments.

Following infection there appears to be a lack of granulocytes in the bone marrow and spleen in all groups. This is surprising as *S. pneumoniae* bacteria induce a strong neutrophil response but this could indicate localisation to the lungs (Kadioglu and Andrew, 2004).

Prior to infection, the immunological profile of mice differed between the two genotypes. Differences observed between the immune cell profile of the two genotypes is contrary to
previous reports showing no difference between the percentages of granulocytes, T and B cells in ApoE−/− mice (Laskowitz et al., 2000). In the periphery of ApoE−/− mice there is a lack of both T and B lymphocytes and granulocytes. This could account for the differences seen in the responses of C57 and ApoE−/− mice to infection. As the ApoE−/− mice did not mount an inflammatory response to infection, they could be less susceptible to a plaque rupture event.

*S. pneumonia* infections at the level used in this study are not sufficient to cause spontaneous stroke however altering the severity of infection may promote a more stroke prone state. We did show a relationship between a systemically driven risk factor for stroke, infection, and the inflammatory environment in the brain. This heightened inflammatory environment could be detrimental if a stroke occurred and indicates a need for infection, as a major risk factor for stroke, to be considered when modelling the disease, in a bid for new treatments.
References


Chapter 5

General Discussion
5.1 Summary of findings

The primary findings in this thesis are summarised below and represented schematically in Figure 8.

1. Both animal models and human patients with risk factors for stroke show neuroinflammation prior to an ischaemic event.

2. Neuroinflammation in atherosclerotic mice is dependent on IL-1 and the use of an IL-1β antibody attenuates both central and peripheral pathology in atherosclerotic mice.

3. Peripheral infection exacerbates cerebrovascular activation and interferes with leukocyte accumulation in atherosclerotic mice.
These findings support the hypothesis that risk factors for stroke have an adverse effect on the inflammatory status of the brain prior to an ischaemic event, which will affect subsequent outcome, and highlights the need for the use of appropriate animal models in developing new treatments for stroke. Findings in chapter 4 identify IL-1β as a potential mediator of neuroinflammatory changes induced by peripheral risk factors and thus as a possible therapeutic target.
5.2 Improving translation from the bench to the clinic

Stroke is a leading cause of death and disability worldwide affecting 111,000 people each year (Scarborough et al., 2009). With an increasing prevalence of risk factors, the number of people affected by stroke will ultimately increase. Even with its devastating consequences, there is still only one treatment for stroke, the clot buster tPA (Khaja and Grotta, 2007). The paucity of available treatments is not simply due to a lack of research. There have been numerous reports of successful agents in preclinical stroke studies, yet none of these agents has demonstrated any efficacy in clinical trial (Scarborough et al., 2009). There has been much discussion to try and explain this lack of translation from the animal stroke studies to man, with increasing focus being placed on the lack of clinically relevant risk factors in most of the preclinical studies completed to date (Fisher et al., 2009). Pre-clinical stroke studies are largely performed in young healthy animals, which is not representative of the patient population (O'Donnell et al., 2010). Stroke patients rarely present without pre-existing conditions such as atherosclerosis, obesity and hypertension and often have multiple risk factors making them co-morbid. Animals with these risk factors show increased ischaemic damage in experimental studies and also respond differently to treatment (Laskowitz et al., 1997; McColl et al., 2010). A possible explanation for this increased damage is that animal models with risk factors for stroke, and indeed patients, have raised levels of inflammation (Ross, 1999; Dandona et al., 2004; Savoia and Schiffrin, 2006). Patients with disorders such as atherosclerosis, obesity and hypertension are now recognised as having raised levels of systemic inflammation, affecting multiple organs, as demonstrated by activation of the acute phase response and higher circulating levels of pro-inflammatory cytokines (Savoia and Schiffrin, 2006; Mathieu et al., 2010; Kablak-Ziembicka et al., 2011). How this raised level of systemic inflammation, in animal models at risk of a stroke, affects the brain has not previously been studied. This thesis highlights that animal models and patients with risk factors for stroke have raised levels of neuroinflammation. It is therefore vital and in accordance with STAIR criteria (Fisher et al., 2009) that potential treatments for stroke are investigated in animal models with risk factors for stroke before they are carried forward for testing in patients.
5.3 Neuroinflammation and risk factors for stroke

The brain was once considered to be immune privileged and separated from systemic inflammation occurring in the periphery. However data from recent years has disproved this and shown the brain to have immunological processes occurring in response to central pathology and also in response to peripheral inflammatory mediators, generated through for example diseases, such as atherosclerosis (chapter 2), and infection (chapter 4) (Galea et al., 2007). Neuroinflammation has been shown to be a key feature in many CNS diseases, acting as both an initiator and also as a consequence of diseases such as stroke (Lakhan et al., 2009). Indeed, post stroke neuroinflammation has been identified as a major contributor to the ongoing ischaemic damage. In this thesis neuroinflammation has been shown through the assessment of vascular activation, microglial activation, in both patients and animal models, and leukocyte infiltration into the choroid plexus. These were determined in areas where a significant difference was seen between risk factor animals and patients and controls. Beyond the existence of neuroinflammation, the functional role of all these inflammatory changes are not known and has to be further investigated. Activated microglial cells were seen adjacent to and around activated VCAM-1 positive blood vessels in the stiatum, as shown in Chapter 3, however the functional role of this is yet to be determined.

5.3.1 Adhesion molecules

Elevations in the levels of adhesion molecules, which are increased in CNS inflammation, have been observed in the CNS in neurological disorders such as stroke and multiple sclerosis and have been shown to correlate with severity of disease (Hu et al., 2010). Their involvement in the infiltration of immune cells such as leukocytes is an important feature in the evolution of damage in stroke (Lindsberg et al., 1996). Pre-clinical stroke studies in which the adhesion molecule ICAM-1 has been removed by both genetic deletion and antibody neutralisation were successful in reducing damage (Clark et al., 1991; Soriano et al., 1996). However, despite these positive findings a clinical trial with the use of an ICAM antibody, Enlimomab, was unsuccessful (Sherman et al., 2001). Chapters 2 and 4 show elevated adhesion molecules in response to both diet induced atherosclerosis and infection in the absence of CNS pathology. These findings highlight adhesion molecules as an important aspect of CNS neuroinflammatory processes and targets which should potentially be revisited as a therapy for stroke. A major criticism of the failed Enlimomab
trial was the antibody used was produced in mice and not humanised. When administered to rats, this antibody did not reduce infarct size and also induced several inflammatory responses including the development of host antibodies, which could account for the lack of effect in the Enlimomab trial (Furuya et al., 2001). Thus, the failure of the Enlimomab trial may have been due to poor experimental design and antibodies against adhesion molecules may still be a viable option for stroke therapy.

5.3.2 Leukocytes

The infiltration of leukocytes in the brain has been shown in neuroinflammatory diseases such as stroke and lupus (Hahn, 1993; Buck et al., 2008). Treatments aimed at reducing leukocytes, namely neutrophil, infiltration, in lupus, showed an alleviation of neuroinflammation was correlated with improved outcome (Farrell et al., 1997). Leukocyte infiltration is also a key stage in the post stroke inflammatory response and has been shown to cause a worsening of damage (Buck et al., 2008). Leukocytes release a wide range of cytokines, chemokines and BBB degrading enzymes and thus promote an inflammatory response. Due to these actions they have been a target in a number of preclinical studies, yet like adhesion molecules, success in animal models of stroke has not been translated to the clinic (Krams et al., 2003). This thesis shows a raised level of leukocytes in the brain in a mouse model of atherosclerosis, a key risk factor for stroke. The promising pre-clinical data, showing increased functional outcomes in stroke following inhibition of leukocytes, and the data from this thesis highlight leukocytes as a continuing target for stroke therapies. A number of criticisms of the acute stroke therapy by inhibition of neutrophils (ASTIN) trial have been made which may account for its failure. Pre-clinical data using this antibody found a reduction in infarct volume in a reperfusion model of stroke and it was not tested in a model of permanent occlusion (Jiang et al., 1998; Zhang et al., 2003). In the ASTIN trial, patients were not monitored for reperfusion. Patients treated with a combination of tPA and the ASTIN antibody showed an improvement in functional outcome, however the trial was not designed to determine the effect of the ASTIN antibody as an adjuvant therapy (Krams et al., 2003). Thus targeting of leukocytes as a therapy for stroke may only be viable in patients with reperfusion. It appears therefore that the failure of the ASTIN clinical trial may be due to poor experimental design and not due to a failure of targeting leukocytes as a neuroprotective agent and this therapy could still remain an attractive target.
5.3.3 Microglial cells

The contribution of microglial cells to the expansion of injury in stroke is controversial and studies show them to have both a beneficial and a detrimental role (Yrjanheikki et al., 1998; Lalancette-Hebert et al., 2007). Microglial cells respond rapidly to pathology in the brain, adopting an activated phenotype that includes upregulation of the expression of cytokines and other inflammatory mediators. These cytokines include the pro-inflammatory IL-1 and TNFα, which are largely believed to be damaging in the ischaemic brain, but also the anti-inflammatory IL-10, which has been shown to have beneficial effects (Hanisch, 2002). The phagocytosis of neutrophils following experimentally induced stroke by microglia is also found to be neuroprotective (Neumann et al., 2008). In this study activated microglia were seen in response to raised levels of peripheral atherosclerotic pathology and this response was sustained in two animal models and also in patients at risk of stroke. Activation of microglia has been shown to be exacerbated in a prion model of chronic neurodegeneration in response to systemic inflammation (Cunningham et al., 2005). Furthermore, in line with increased microglial priming, a worsening of cognitive impairment is seen in animal models of delirium (Murray et al., 2011). Therefore, although the precise role microglia play in the progression of ischaemic damage at any one time is disputed, the presence of activated microglia as a hallmark of a disruption to normal brain homeostasis is not in doubt.

Differences were seen in the levels of microglial activation between those ApoE−/− mice fed a Paigen diet in chapters 2 and 3 and those in chapter 4. Mice from chapters 2 and 3 were bred and housed with collaborators however mice from chapter 4 were bought form external sources and raised locally. There may be slight genetic variations between these sets of mice which had a larger implication in the responsiveness of microglial cells to the development of atherosclerosis. As such, it would be interesting to determine the exact genetic sequences of these mice to establish any differences which could indicate why there was a limited response of microglial cells in chapter 4 to the development of atherosclerosis. Mice from chapter 4 did show increased leukocyte infiltration and increased vascular activation and therefore microglial activation may not be a sensitive measure of neuroinflammation. Microglial cells reside in the parenchyma of the brain whereas blood vessels and the choroid plexus are more likely to be exposed to peripherally derived inflammatory mediators.
The vascular activation, leukocyte infiltration and microglial activation shown in this thesis demonstrate that neuroinflammation can occur in response to peripherally driven pathology. The data also suggests that atherosclerosis, a major risk factor for stroke, could prime both the peripheral and central inflammatory response and therefore lead to a worsened outcome following an ischaemic event. Therefore vascular activation, leukocyte infiltration and microglial activation remain attractive targets for therapies in stroke research.

5.4 IL-1 as a target in disease

Blockade of IL-1α/β signalling by genetic deletion, antibodies and the endogenous ligand IL-1Ra has proved a successful approach in the reduction of disease pathology in several experimental models of disease, including atherosclerosis, arthritis and stroke (Denes et al., 2011). Clinically this has translated to the use of IL-1Ra in rheumatoid arthritis and anti-IL-1β antibodies in the Cryopyrin Associated Periodic Syndromes (Bresnihan et al., 1998; Lachmann et al., 2009). In relation to CNS disease IL-1Ra has completed a small phase 2 trial in acute ischaemic stroke (Emsley et al., 2005). The findings in this thesis highlight IL-1 as a key link between peripheral atherosclerosis and neuroinflammation. Indeed, IL-1 may also prove important in providing a link between the systemic inflammation seen in other situations, such as with infection, and subsequent neuroinflammatory changes. As such these observations reinforce the potential of IL-1 as a viable therapeutic target.

5.4.1 Anti-IL-1β antibody as a treatment in stroke

The beneficial effect of IL-1 blockade, as shown in transgenic mice lacking IL-1α and/or IL-1β (Boutin et al., 2001) and also through the use of the endogenous antagonist IL-1Ra (Relton and Rothwell, 1992), in stroke makes it an attractive target for treatment. In this thesis an IL-1β antibody reduced vascular activation and leukocyte infiltration in the brain, both of which have been shown to be detrimental in the outcome following stroke. The use of an antibody that inhibits multiple phases of the inflammatory response, as this one does, may provide a beneficial outcome. Another advantage of the IL-1β antibody is that it had no effect on microglial activation. In stroke studies where microglial proliferation has been inhibited, animals showed a worse outcome and literature suggests a neuroprotective role of microglial cells following ischaemia (Lalancette-Hebert et al., 2007). Therefore this
antibody could allow beneficial host defences to remain intact and target only those with a negative impact on outcome.

Both prior to and following stroke, inflammatory processes occur which need to be accounted for in the search for treatments. These inflammatory components add to the complex cascade of events which lead to cell death. With such complex interactions occurring, it seems unlikely that one treatment alone will be able to attenuate the damage occurring following stroke and the multiple failed clinical trials seem to support this theory. A combinatorial therapy strategy may provide an avenue for treatment development. In ischemic stroke, reperfusion appears to be a key step in aiding recovery however it is at this time that inflammatory mediators have rapid access to induce damage. Use of an IL-1β antibody in combination with for example tPA could allow necessary reperfusion to occur but could also provide a reduction in the inflammatory response which increases neuronal death.

Ideally treating the risk factors for stroke as a primary prevention would be most effective as it could lead to a marked reduction in the incidence of stroke and another use for the IL-1β antibody is in the treatment of atherosclerosis. It has previously been reported that an IL-1β antibody attenuates the development of atherosclerosis in ApoE<sup>−/−</sup> mice, though its effects on neuroinflammation are unknown (Bhaskar et al., 2011). Developing treatments for atherosclerosis is important as rupture of atherosclerotic plaques is a major source of occluding thrombi, thus removal could entirely prevent stroke occurrence. The dual features of this antibody provide an attractive treatment as it can not only reduce atherosclerosis but also attenuate neuroinflammation. However the use of an IL-1β antibody long term to achieve attenuation of risk factors may not be possible as long term immunosuppression is known to induce a number of side effects including kidney failure and cancer (Abraham and Thompson, 2010). Secondary effects of the IL-1β antibody, such as reduced hepatic inflammation, highlight it as a possible treatment for other diseases. Studies observing the effect of acute IL-1β antibody treatment in atherosclerosis and neuroinflammation need to be considered to determine the time frame needed for reduced peripheral pathology and neuroinflammation. However as an initial pre-clinical study these results are promising.

5.4.3 IL-1 and decreased motivational disorders

IL-1 signalling has been closely linked with the development of decreased motivational disorders such as depression (Koo and Duman, 2009; Diniz et al., 2010; Lehto et al.,
The results in this thesis highlight a role for the use of IL-1 in the treatment of these decreased motivational disorders, as increased motivation was observed in IL-1R1\textsuperscript{+/−}/ApoE\textsuperscript{−/−} mice compared with ApoE\textsuperscript{−/−} mice fed a Paigen diet. Although anti-depressant treatments are available, some people remain resistant and suffer long term depression which is not widely understood (Trivedi et al., 2007). A possible link between neuroinflammation and depression has been developed and this is an area for continued research (Alexopoulos and Morimoto, 2011). There are currently increases in both poor diet and depression in society, with the model chosen in this thesis being one of diet induced atherosclerosis, a new model may have been found to study the effects of poor diet on motivational disorders and also a role of IL-1 as a link between the two. The systemic leukocytes which are recruited to the brain in response to diet induced atherosclerosis may be a potential source of IL-1 (Konsman et al., 2007) which can cause an activation of resident IL-1 producing cells in the brain (Tsakiri et al., 2008) and also induce neuroendocrine changes (Curti et al., 1996) all of which are known to exacerbate depressive behaviours (Koo and Duman, 2009; Zunszain et al., 2011).

5.5 Future directions to reduce stroke occurrence and outcome

The availability of imaging techniques allowed us to show the suitability of the animal models chosen to reflect patients with risk factors for stroke. Studies in multiple sclerosis have also shown a translation between animal models and patients, using imaging techniques to show similarities in leukocyte infiltration and accumulation (Kent et al., 1995; Moll et al., 2009). These imaging techniques could therefore be more heavily adopted in stroke research to gain a better understanding of the development of injury post stroke and may aide in the identification of novel treatments. Developing a screening technique for patients who are most at risk of stroke could lead to increased monitoring and thus more rapid treatment if a stroke did occur. The 4.5 hour time window for the use of tPA is a major barrier in its use and this is attributed to the need for imaging of patients, for cerebral aneurysms and haemorrhagic stroke, prior to its use. Developing new therapies or those which would extend this time window are therefore of key importance.

Treatment of neuroinflammatory changes associated with risk factors is not currently performed however further study into the effects of reducing neuroinflammation could be an interesting avenue of research. Reducing these neuroinflammatory changes,
through the use of antibodies or modifications in lifestyle, could prevent exacerbated ischaemic damage if a stroke occurred.

In addition to using appropriate animal models to find treatments post stroke, it seems vital that steps are taken to properly manage risk factors for stroke in patients. Diseases such as obesity, hypertension and atherosclerosis are common and treatable in many cases. Identifying and treating patients with risk factors associated with stroke could ultimately lead to a decrease in the prevalence of the disease. One proposed method is the ‘polypill’ approach where everyone over the age of 55 for example is prescribed a single preparation containing multiple drugs to treat multiple risk factors. A study with a preparation to modify LDL cholesterol, blood pressure, homocysteine, and platelet function predicted a decrease in stroke prevalence by 80% (Wald and Law, 2003). Over the last ten years the incidence of stroke has fallen and this is likely to have been caused by the increased treatment and prevention of risk factors such as hypertension and atherosclerosis through the use of for example anti-hypertensive and lipid lowering drugs (Lee et al., 2011).

5.6 Future work

The identification of neuroinflammation in both patient and animal models of risk factors for stroke is an interesting finding. To further the work a number of experiments could be performed.

*How would an IL-1β antibody affect infarct volume and functional outcome in an experimental model of stroke?*

Administration of the endogenous ligand IL-1Ra is known to decrease lesion volume and increase functional outcome (Relton and Rothwell, 1992), however no data is available on the effects of the IL-1β antibody used in this thesis. In accordance with the STAIR criteria (Fisher et al., 2009), the IL-1β antibody should be tested in an animal model with risk factors for stroke such as the ApoE<sup>-/-</sup> mouse or the corpulent rat. These animals will therefore have systemic inflammation which is known to adversely effect outcome following stroke. The antibody should be administered in a clinically relevant manner and therefore administering at the earliest at 3 hours following ischaemia. To
maintain clinical relevance, a longitudinal study should be performed with regular imaging to assess the development of lesions.

Another experiment that could be performed would be to administer the antibody prior to onset of stroke and determine how the lesion volume and functional outcomes are effected. If patients at immediate risk of stroke could be identified then it would be beneficial to be able to treat these patients not only for the risk factors they have but also with a neuroprotective agent that could limit any damage occurring in an ischaemic event.

*Is decreased CNS inflammation a result of decreased peripheral pathology or due to blockade of inflammatory signals to the brain?*

A key area to be explored in this study is determine whether the decreases in neuroinflammation is in response to reduced IL-1 actions or is due to the reduction in peripheral atherosclerosis. To do this, firstly ApoE\(^{-/-}\) mice could be crossed with mice that have a brain specific deletion of IL-1R1 such as on astrocytes or microglial cells. The mouse model would initially need to be assessed for the presence of atherosclerotic lesions to ensure the peripheral inflammatory pathology is present. Secondly the levels of neuroinflammation would be assessed. If the mice lacked neuroinflammation in the brain then it would be possible to say that neuroinflammation arising from peripheral pathology is dependent on IL-1 signalling, as this mouse has IL-1R1 only removed from the brain specific cells. There may however be inflammatory mechanisms which do not occur through the specific cells with an IL-1R1 deletion and thus an animal model with IL-1R1 removed from all cells of the brain would be ideal however this would be a complex model to develop.

*Does S. Pneumonia induce plaque rupture and could a change in the infection protocol induce spontaneous strokes?*

In chapter 4 animals were infected with *S. pneumoniae* however no spontaneous stroke events were observed. In this study, the effect of *S. Pneumonia* on peripheral atherosclerotic plaque pathology were not studied therefore it would be interesting to determine whether *S. Pneumonia* has a similar effect on plaques as seen with *C. Pneumoniae* (Ezzahiri et al., 2003). To identify changes in plaque pathology, the experiment could be repeated and cross sections of the aorta could be stained with oil red o to determine lesion area and a fibrinogen antibody used to determine the integrity of the
fibrous cap. The infection protocol may also need to be altered and an increased dose or increased time of infection may lead to spontaneous stroke in these mice.

Does an IL-1β antibody increase motivational behaviour in atherosclerotic ApoE<sup>−/−</sup> mice?

Atherosclerotic mice showed depressive behaviour which was attenuated in ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup> mice. However the effect of an IL-1β antibody on the motivational behaviour of animals was not tested. The IL-1β antibody study could be repeated and the motivational behaviour assessed through a similar wheel running experiment used on the ApoE<sup>−/−</sup>/IL-1R1<sup>−/−</sup> mice. Assessing whether patients with either atherosclerosis or a poor diet have symptoms of depression would also be interesting and this could be done by looking through case-notes and also assessing existing patients. High fat diets have previously been shown to raise the levels of depression in rats (Abildgaard et al., 2011) and with a society with increasingly poor dietary habits, depression may rise and the actions of the IL-1 may provide a good route for treatment. It would be interesting to discover the anti-depressive properties of this IL-1β antibody using standard depression tests such as the forced swimming test. If these experiments prove successful then this antibody may be a possible new route for therapy.


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Chapter 6

Supplementary Methods
7.1 Solutions

0.4M PB buffer:
Dissolve 57.29g disodium hydrogen orthophosphate dihydrate (Na$_2$HPO$_4$·2H$_2$O) (Fischer, UK) and 11.035g Sodium dihydrogen orthophosphate hydrate (NaH$_2$PO$_4$·H$_2$O) (Fischer, UK) in 1L dH$_2$O.

4% PFA
Heat 600 ml dH$_2$O to 60°C in flask in fume hood and add three granules NaOH (Sigma, UK) to allow PFA powder (Sigma, UK) to dissolve. Add 40g PFA powder continue stirring and maintain heat until dissolved. Add 250ml 0.4M PB and top up to 1L with dH$_2$O. Filter in fume hood with Millipore pump and set the pH to 7.4.

Primary diluent (immunohistochemistry buffer)
Heat 40ml 1xPBS to 40/50°C and dissolve 600µl of Triton into it. Top up with 160ml of cool PBS and add 20mg of sodium azide (Sigma, UK) to preserve.

0.2M Sodium Acetate
Add 1.641g of anhydrous sodium acetate (Fischer, UK) to 100ml of dH$_2$O. Set pH 6.0 using glacial acetic acid (Fischer, UK).

Nickel ammonium sulphate
Dissolve 1.5g nickel ammonium sulphate (Sigma, UK) in 50ml of 0.2M sodium acetate.

0.9% Saline
Dissolve 9g sodium chloride (Fischer, UK) in 1L dH$_2$O.

Glycerol Jelly mounting medium
Add 10g Gelatine powder (Fischer, UK) to 60ml Water, dissolve by warming and stirring. Add 70ml Glycerol (Fischer, UK) and mix gently to avoid inclusion of air bubbles. Add 2 drops of liquid phenol (Fischer, UK). Store at 4°C. **To cover slip** Cover slide with mounting medium slowly to prevent air bubbles and cover slip. Leave slide in warm (40°C - 45°C) for 30 minutes to allow medium to enter tissue. Check for air bubbles if not ok melt mount under warm water and repeat mounting. Place in fridge to set.
**Cryoprotectant solution**

Dissolve 6.6g disodium hydrogen orthophosphate dihydrate (Na$_2$HPO$_4$ * 2H$_2$O) (Fischer, UK) and 0.79g sodium dihydrogen orthophosphate hydrate (NaH$_2$PO$_4$ * H$_2$O) (Fischer, UK) in 500ml dH$_2$O. Then add 300ml Ethylene glycerol, anhydrous (Sigma, UK) and 200ml glycerol (Fischer, UK), mix well. Can be stored at room temperature.

**Oil Red O stain**

To make 100ml of ORO (if using square staining jars make 300ml) add 0.25g ORO powder (Sigma, UK) to 67ml Isopropranol (Fischer, UK), stir for 10 minutes. Add 33ml dH$_2$O and stir for a further 1 minute. Leave to stand for 6 minutes and filter. Rinse mounted sections in dH$_2$O and transfer to 60%v/v isopropranol for 30 seconds. Incubate sections in ORO stain for 18 minutes, place in 60%v/v isopropranol for 30 seconds and rinse in dH$_2$O.

**Scotts tap water solution**

Dissolve 3.5g sodium bicarbonate (Sigma, UK) and 20g magnesium sulphate (Sigma, UK) in 1L dH$_2$O.

**Homogenisation buffer**

Dissolve 7.88g TRIS-HCL (Fischer, UK), 8.76g NaCl (Fischer, UK), 0.55g CaCl$_2$ (Fischer, UK) and 0.2g NaN$_3$ (Sigma, UK) to 1l dH$_2$O. Set pH to 7.4.

**ACK buffer**

Dissolve 8.29g NH$_4$Cl (Fischer, UK), 1.0g KHCO$_3$ (Fischer, UK) and 0.0367g Na$_2$-EDTA (Fischer, UK) to 1l dH$_2$O. Adjust pH to 7.4 with IM HCl.

**7.2 Flow cytometry cell labelling**

**7.2.1 Blood cells**

Following sampling, blood was placed on ice and cells were labelled within 2 hours. Whole blood (25µl per labelling group) was aliquotted and the Fc Receptor was blocked (0.5µl FC block) for 20 minutes. Blood cells were then labelled with the appropriate
concentrations of antibody (see table below, all antibodies from eBioscience, UK) for 30 minutes.

<table>
<thead>
<tr>
<th></th>
<th>Antibody</th>
<th>Concentration</th>
<th>Conjugation</th>
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<tbody>
<tr>
<td><strong>Cocktail 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td></td>
<td>1:200</td>
<td>FITC</td>
</tr>
<tr>
<td>CD8</td>
<td></td>
<td>1:400</td>
<td>PE</td>
</tr>
<tr>
<td>CD14</td>
<td></td>
<td>1:100</td>
<td>APC</td>
</tr>
<tr>
<td><strong>Cocktail 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD19</td>
<td></td>
<td>1:100</td>
<td>FITC</td>
</tr>
<tr>
<td>Ly6G</td>
<td></td>
<td>1:400</td>
<td>PE</td>
</tr>
<tr>
<td>MHCII</td>
<td></td>
<td>1:500</td>
<td>APC</td>
</tr>
</tbody>
</table>

Red blood cells were then lysed with a 1x lysis solution for at least 10 minutes and the samples read on an available flow cytometry machine.

### 7.2.2 Bone marrow and spleen cells

Following extraction, spleens were homogenised and placed in FACs buffer. The left hind leg was removed and both the tibia and fibula were flushed with FACS buffer to obtain the bone marrow cells. Samples were placed on ice in FACs buffer and labelling began within 2 hours. Samples were spun in a centrifuge for 8 minutes at 400g and the supernatant was discarded and the pellet disrupted. Red blood cells were depleted using ACK buffer for 1 minute and then 10ml of PBS was added. The samples were spun in a centrifuge for 8 minutes at 400g and the supernatant was discarded and the pellet disrupted. After adding 2ml of FACs buffer to each sample, they were filtered through 80µm nylon mesh by pipetting with a 5ml pipette. 200µl of each sample was loaded into a 96 round bottomed well plate and samples were spun for 3 minutes at 400g. Fc receptors were blocked (30µl per well of 1:200 FC block) for 30 minutes, 200µl of FACs buffer was added to each well and samples were spun for 3 minutes at 400g. Antibodies were diluted (table below, all antibodies were sourced from eBioscience, UK) and 30µl of cocktail was added to each well, plates were covered with foil and placed on an orbital shaker for 30 minutes.
<table>
<thead>
<tr>
<th></th>
<th>Antigen</th>
<th>Dilution</th>
<th>Fluorochrome</th>
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<tr>
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<td>CD3</td>
<td>1:200</td>
<td>FITC</td>
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<tr>
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<td>CD8</td>
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<td></td>
<td>CD49b</td>
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<td>APC</td>
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</tr>
<tr>
<td></td>
<td>MHCII</td>
<td>1:500</td>
<td>APC</td>
</tr>
</tbody>
</table>

200µl of FACs buffer was added to each well and samples were spun for 3 minutes at 400g, wells were topped up with 200µl of FACs buffer and read on an available flow cytometry machine.

### 7.2.3 Cell population gates

Cell populations shown in this thesis were gated as follows:
Figure 1. Gating strategy for flow cytometry cell populations. A) the same gating strategies were used for bone marrow and spleen cell populations CD3, CD4, CD19 identified using a FITC conjugated antibody. CD11b and CD8 were identified using a PE conjugated antibody. B) Gating strategy for blood cells, both CD3 and CD8 were measured using a FITC conjugated antibody.

### 7.3 Histology

#### 7.3.1 Haematoxylin and Eosin

Sections were mounted onto gelatine coated slides, hydrated through 100%, 90% and 70% alcohols for 2 minutes each and transferred to dH₂O. Slides were transferred to Haematoxylin for 5 minutes, rinsed in dH₂O, transferred to Scotts tap water for 30s and again rinsed in dH₂O. Following this slides were placed in Eosin for 30s, rinsed in dH₂O, dehydrated through 70%, 90% and 100% alcohol for 2 minutes each, placed in xylene for 30 minutes and finally coverslipped with DePex.
7.4 **CBA**

7.4.1 **Tissue preparation**

Spleen and liver tissue were homogenised using a small hand held homogeniser (Kontes, USA) and an ultrahomogeniser (IKA, USA) respectively. All tissue was homogenised, until no visible pieces remained in 250µl homogenisation buffer per 50mg tissue, and left on ice for 30 min. Homogenate was then centrifuged (17,000 x g, 30 min, 4°C) and pellet discarded. Protein assays were then immediately carried out on supernatants to avoid multiple freeze-thaws.

7.4.2 **Protein assay**

Total protein concentrations were calculated using a bicinchoninic acid (BCA) protein assay (Pierce, USA). Bovine serum albumin (BSA) was used as the standard against which the concentration of protein in each sample was determined. A series of dilutions of BSA (800ng/ml to 12.5ng/ml) diluted in PBS was used to generate a standard curve. Sample was diluted (1:50 for spleen and 1:100 for liver in PBS) and vortexed. 50µl of each diluted sample and standard was then added in duplicate to a 96-well plate and 200µl of BCA working reagent, prepared by mixing 50 parts reagent A (containing BCA) with 1 part reagent B (containing cupric sulphate), to each well. This was then incubated for approximately 30min at 37°C. The purple coloured product is proportional to the total amount of protein. Absorbance was read at a wavelength of 570nm on a plate reader (Dynatech Laboratories, USA) and the concentration of samples determined from the standard curve.

7.4.3 **CBA assay**

CBA was carried out according to the manufacturer’s instructions but with a 5 fold reduction in the recommended volumes added. Standards were prepared by combining the lyophilised standard spheres provided for each flex set, reconstituting in 4ml assay diluents and performing serial dilutions to create a 10 point standard curve. 10µl of each sample was added to a round ended well of a 96-well plate (CoStar, UK) then 10µl of a solution containing all capture beads was added to each well. Each plate was then incubated for 1 h on a shaker before 10µl of a solution containing mixed detection reagent was added. Plates
were incubated on a shaker in the dark and then plates were centrifuged (eppendorf, Germany), supernatant was removed and beads re-suspended in 140μl of wash buffer. Samples were then acquired on a flow cytometer using BD FACSCompTM software (BD biosciences, UK).

7.5 Paigen Diet

The Paigen diet is provided in powder form (Special diet services, UK – 829110 atherogenic Paigen diet) and therefore needs to be reconstituted. Lumps were initially removed and then 1.5kg of powder was mixed well, with 300ml of cold tap water, to create a wet paste. This was then distributed evenly on baking trays which had been covered with enough foil to cover the edges of the diet to prevent any oil leakage. The diet was then dehydrated in an oven at 100°C for 3 hours until the diet had a golden brown appearance, allowed to cool and placed in a 4°C fridge overnight to solidify. For a cage of 5 mice approximately 30g of diet was fed per day, however this quantity may need to be increased initially as mice adjust to the consistency of the diet.