The role of platelet-derived interleukin-1 alpha as a driver of neutrophil migration *in vivo*.

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

2012

James Giles
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<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ALI</td>
<td>acute lung injury</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AP</td>
<td>air pouch</td>
</tr>
<tr>
<td>ASC</td>
<td>apoptosis-associated spec-like protein with a CARD domain</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BALF</td>
<td>broncho-alveolar lavage fluid</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid assay</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>cellular adhesion molecule</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBA</td>
<td>cytometric bead array</td>
</tr>
<tr>
<td>CCL</td>
<td>chemokine (CC subtype) ligand</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>cyclic oxygenase</td>
</tr>
<tr>
<td>CX3CL</td>
<td>chemokine (CX3C subtype) ligand</td>
</tr>
<tr>
<td>CXCL</td>
<td>chemokine (CXC subtype) ligand</td>
</tr>
<tr>
<td>DAMP</td>
<td>damage associated molecular pattern</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMLA</td>
<td>eutetic mixture of local anaesthetic</td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>Gp</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>IL-1R1</td>
<td>interleukin-1 receptor 1</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IL-1RAcP</td>
<td>interleukin-1 receptor accessory protein</td>
</tr>
<tr>
<td>KC</td>
<td>keratinocyte-derived cytokine (aka CXCL1)</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBEC</td>
<td>mouse brain endothelial cell</td>
</tr>
<tr>
<td>MP</td>
<td>microparticle</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>ND50</td>
<td>50% neutralisation dose</td>
</tr>
<tr>
<td>NET</td>
<td>neutrophil extracellular trap</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NLRP</td>
<td>NOD-like receptor family, pryin domain containing</td>
</tr>
<tr>
<td>NOD</td>
<td>nucleotide organisational domain</td>
</tr>
<tr>
<td>ns</td>
<td>non-significant</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PB</td>
<td>phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PECAM</td>
<td>platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear granulocyte</td>
</tr>
<tr>
<td>PSGL1</td>
<td>P-selectin glycoprotein ligand 1</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated upon activation, normal T-cell expressed, and</td>
</tr>
<tr>
<td></td>
<td>immunity related gene</td>
</tr>
<tr>
<td>RD</td>
<td>reagent diluent</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SSC</td>
<td>side scatter</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TXA₂</td>
<td>thromboxane A₂</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VVO</td>
<td>vesicu-lo-vacuolar organelle</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>XCL</td>
<td>chemokine (XC subtype) ligand</td>
</tr>
</tbody>
</table>
The role of platelet-derived interleukin-1 alpha as a driver of neutrophil migration in vivo.

Neuroinflammation is an important contributor to the pathogenesis of many neurological diseases. A key component of the innate immune response in the central nervous system is the migration of neutrophils into the brain parenchyma, where they exacerbate neuronal injury and worsen clinical outcome. A greater understanding of the mechanisms underlying neutrophil influx into the brain may aid the development of novel therapeutic interventions for the variety of diseases to which neutrophils contribute, notably including stroke and epilepsy. In vitro evidence implicates the pro-inflammatory cytokine, interleukin-1α (IL-1α), derived from platelets as a key mediator of cerebrovascular inflammation and neutrophil migration across brain endothelial cells. The aim of the work in this thesis was to test if this mechanism is important in vivo.

We investigated the contribution of platelets and IL-1 in a murine model of neutrophil migration into the peritoneal cavity in response to injection of lipopolysaccharide (LPS). Depletion of platelets abrogated the migration of neutrophils in response to LPS-induced peritonitis, indicating an important role for platelets in the process. Genetic knockout of IL-1 had no effect on neutrophil influx, demonstrating that migration in the peritoneum occurs independently of IL-1.

The discovery that neutrophil migration in LPS-induced peritonitis was independent of IL-1 contrasted with the finding that platelet-derived IL-1 was a mediator of neutrophil influx across mouse brain endothelial cells in vitro. The question arose as to whether IL-1 was required as a mediator of neutrophil migration in extra-cerebral tissues. Hence, we tested the contribution of platelets and IL-1 in two further in vivo models of neutrophil migration: LPS injection into a subcutaneous air pouch, and acute lung injury induced by LPS inhalation. Platelet depletion significantly reduced neutrophil migration into the air pouch in response to LPS, yet had no effect in acute lung injury. This indicated that neutrophil migration into the air pouch was dependent on platelets, and that migration into the lungs was platelet-independent. LPS induced the same degree of neutrophil migration in wild-type and IL-1 knockout mice, demonstrating that IL-1 was not required for neutrophil migration in either model.

To determine the contribution of platelets and IL-1 to neutrophil migration in response to cerebrovascular inflammation, we injected LPS into the mouse striatum. In this model, neutrophil influx to the brain parenchyma in response to LPS was reduced by depletion of circulating platelets, and inhibition of the platelet adhesion molecule, GpIb. Genetic knockout of IL-1α significantly reduced the number of invading neutrophils induced by LPS. These data confirmed that both platelets and IL-1α were important contributors to cerebral neutrophil migration in vivo. To determine whether platelets in systemic circulation may be the source of IL-1α, we treated mice with IL-1 receptor antagonist or anti-IL-1 antibodies to block systemic IL-1 action. Neither intervention affected cerebral neutrophil migration in response to LPS, suggesting that the IL-1α that mediates neutrophil migration may originate in the brain.

Overall, these data demonstrate that IL-1α and platelets make an important contribution to neutrophil migration to the brain, yet independently of each other. Our data also suggest there may be specific mechanisms driving innate immune responses in vivo even in response to the same inflammatory stimulus.

James Anthony Giles
PhD Neuroscience, The University of Manchester

7 November 2012
v. Declaration

No portion of the work referred to in this 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.

James Giles, 7 November 2012

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vii. Acknowledgements

I remember the response when I first approached my MRes supervisor, Stuart Allan, about wanting to convert to a PhD programme. He was surprised, but interested. He subsequently guided me through the challenge of getting on the programme, developing my experiments and finishing my thesis. He is an inspiring scientist, an excellent teacher, and a superbly helpful mentor. I couldn’t have completed the programme without Stuart, and I am immensely grateful to him for giving me the space to develop Fastbleep in parallel to my PhD; not all supervisors would have understood, and Stuart’s influence is clear in Fastbleep’s widening participation work with schools.

I also remember Barry McColl’s response when I told him I couldn’t use a micropipette as I started the MRes. He was also surprised, to put it mildly. His immense patience prevailed though, and the data his supervision helped me accrue in my first year meant I was able to transfer to the PhD, and Barry’s detailed redrafting of my funding proposals ensured their success. No scientist I’ve ever worked with has paralleled Barry’s talent for explaining and applying principles of science in day-to-day experiments.

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I thank the Faculty of Medical and Human Sciences for funding my first year, the Faculty of Life Sciences for bridge funding, and the British Heart Foundation for funding my PhD conversion via their MBPhD studentship scheme.
Chapter 1

Introduction
1.1 Overview

Inflammation in the brain (i.e. neuroinflammation) contributes to a variety of neurological disorders. An acute insult to brain tissue, such as ischaemic stroke, causes cell death and initiates an inflammatory response (Allan et al. 2005). Inflammatory processes occur at both a local and systemic level and, in this context, exacerbate the initial brain injury. An integral part of the cerebral inflammatory response is the migration of neutrophils from the circulation into brain tissue, and blocking neutrophil migration may improve neurological outcome (Ransohoff & Brown 2012). Currently, we have limited experimental data elucidating the mechanisms underlying neutrophil migration in the central nervous system (CNS). This introduction provides an overview of neuroinflammation, its chemical and cellular mediators, and the mechanisms and pathways through which they interact.

1.2 Inflammation in the CNS

Inflammation occurs after recognition of pathogens or cell death, and is classically defined in terms of four cardinal signs: heat, pain, redness, and swelling (Celsus 25AD). Primarily, inflammation is an organism’s response to protect against the harmful effects of pathogens or damaged cells, to remove such stimuli, and to initiate healing. Over the last twenty-five years, the classical view of the CNS as an ‘immune-privileged’ organ has been gradually deconstructed. It was previously believed that the brain was neither susceptible nor contributory to inflammation (Lucas et al. 2006). It has now been demonstrated that inflammation in the CNS occurs in response to a wide variety of pathological stimuli, both acutely, as in trauma, infection and stroke, and in more chronic conditions such as Alzheimer’s and Parkinson’s diseases (Allan et al. 2005). Cardinal components of neuroinflammation include alterations in local blood flow, leukocyte infiltration (including neutrophils) and the activation of microglia, astrocytes and endothelial cells. For example, following an ischaemic insult in the CNS, activation of microglia and release of inflammatory mediators occurs rapidly within minutes, while leukocyte recruitment occurs over subsequent hours (Lucas et al. 2006; Lambertsen et al. 2012).
Inflammatory pathways may be categorised as sterile or non-sterile according to the trigger of inflammation. Non-sterile triggers of inflammation include bacterial, fungal and viral infection, where the presence of pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS), initiates the inflammatory response. The recent discovery of pattern recognition receptors (PRRs), and notably for LPS, toll-like receptors (TLRs), as the molecular detectors of PAMPs instigated new interest in the field of innate immunity (Medzhitov 2001). Since then, sterile inflammation has been shown to involve damage-associated molecular patterns (DAMPs) that trigger inflammation. In healthy cells, DAMPs remain hidden from the innate immune system in the cellular interior (Kono & Rock 2008). Following an insult, for example ischaemic or traumatic cellular injury, or apoptosis, cells may become necrotic, leading to the release of DAMPs, which may include DNA, ATP and intracellular proteins (Chen & Nuñez 2010). Cerebral inflammation involves a complex interplay of cellular and molecular mediators from a diverse range of organs, including the CNS, systemic circulation, bone marrow, and liver. The interface of these two compartments, the blood-brain barrier (BBB), plays a crucial role in the inflammatory process.

1.3 The blood-brain barrier
The BBB is a specialised, selectively permeable layer of endothelial cells that line CNS blood vessels (Abbott et al. 2006). Properties of the BBB are regulated by complex interactions between the cellular constituents of the neurovascular unit, which comprises endothelial cells, perivascular cells, astrocytes and neurones (Figure 1.1) (Abbott et al. 2006). The BBB is selectively permeable due to tight junctions that exist between endothelial cells, thus, under normal conditions, substances pass via a tightly-regulated transcellular route (Wolburg & Lippoldt 2002; Hawkins & Davis 2005). This prevents cells, such as leukocytes, or proteins from crossing the BBB, while small molecules such as O₂, CO₂ and ethanol pass freely (Abbott et al. 2006). During CNS inflammatory responses, circulating leukocytes enter the brain at a slower rate compared to other tissues (Ransohoff & Brown 2012). Entry is enhanced by both cerebrovascular inflammation and BBB disruption (del Zoppo et al. 2000), which has been observed in a variety of CNS conditions, including stroke, epilepsy and

Figure 1.1 – Components of the blood brain barrier
The BBB exists between the circulating blood and the brain parenchyma and is formed by endothelial cells and their tight junctions, the basement membrane, pericytes, astrocytes and neurones. For neutrophils to migrate into the brain parenchyma, they must migrate across the endothelial monolayer, through the basement membrane and through the cerebral extracellular matrix.

1.4 Cytokines
Cytokines are key molecular mediators of inflammation, including cerebrovascular inflammation (Thornton et al. 2010). The term encompasses a large number of proteins that exert a diverse range of effects. Usually less than 20kDa in size (Wood 2001), cytokines comprise a number of families, including the interleukins, interferons, tumour necrosis factors, colony-stimulating factors, growth factors and chemokines (Table 1.1).
The term chemokine is a contraction of ‘chemotactic cytokine’. These molecules are released by a variety of immune cells. The chemokine family can be further divided into four subgroups based on the arrangement of cysteine residues on their N-terminal region (Le et al. 2004). These molecules act as attractants for immune cells, directing them to a specific site. More recent studies have indicated a role for chemokines in regulating homeostatic mechanisms, particularly T-cell development (Bachmann et al. 2006). Notable chemokines include CXCL1 (KC), which is a strong recruitment signal for neutrophils, and CCL5 (RANTES), which attracts T-cells, eosinophils and basophils.

<table>
<thead>
<tr>
<th>Family</th>
<th>Subgroup</th>
<th>Example</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukins</td>
<td>IL-1 family</td>
<td>IL-1</td>
<td>Pro-inflammatory effects, induction of cytokines; cell differentiation, proliferation, apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-33</td>
<td>Pro-inflammatory, induction of cytokines</td>
</tr>
<tr>
<td></td>
<td>Haematopoetins</td>
<td>IL-2</td>
<td>Differentiation and proliferation of lymphocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-6</td>
<td>Regulation of lymphocytes, haematopoiesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-10</td>
<td>Anti-inflammatory, regulation of T-cells</td>
</tr>
<tr>
<td>Interferons</td>
<td></td>
<td>IFN-γ</td>
<td>Anti-viral, regulation of lymphocyte development</td>
</tr>
<tr>
<td>Tumour necrosis factors</td>
<td></td>
<td>TNF-α</td>
<td>Cytotoxic, proliferation and differentiation of many cell types</td>
</tr>
<tr>
<td>Transforming growth factors</td>
<td></td>
<td>TGF-β</td>
<td>Healing, regulation of cell proliferation, regulation of inflammation</td>
</tr>
<tr>
<td>Colony-stimulating factors</td>
<td></td>
<td>G-CSF</td>
<td>Regulation of granulocyte proliferation and differentiation</td>
</tr>
<tr>
<td>Chemokines</td>
<td>CC</td>
<td>CCL5 (RANTES)</td>
<td>Chemotactic for T-cells, eosinophils, basophils</td>
</tr>
<tr>
<td></td>
<td>CXC</td>
<td>CXCL1 (KC)</td>
<td>Chemotactic for neutrophils</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>XCL1 (lymphotactin)</td>
<td>Chemotactic for T-cells, B-cells</td>
</tr>
<tr>
<td></td>
<td>CX3C</td>
<td>CX3CL1 (neurotactin)</td>
<td>Chemotactic for T-cells, monocytes</td>
</tr>
</tbody>
</table>

Table 1.1 – Cytokine families and subgroups. Examples of different cytokines are given within each subgroup, with their principle effects. Adapted from Oppenheim & Hopkins 2001; Paul 2008.
1.5 Interleukin-1 in neuroinflammation

Interleukin-1 (IL-1) is a pro-inflammatory cytokine. It was first discovered in the 1940s and described as ‘endogenous or leukocytic pyrogen’ serving as a link between fever, infection and inflammation (Dinarello 2008). Since then, eleven members of the IL-1 family have been identified. IL-1 itself comprises two ligands, IL-1α and IL-1β, both 17kDa proteins. Though produced from different genes, these proteins have high sequence homology and exert similar effects (Allan et al. 2005).

IL-1 is produced by a diverse range of cell types, including neutrophils, monocytes, macrophages, dendritic cells, B-cells, T-cells, microglia, platelets, endothelial cells and dying cells (Sims & Smith 2010). During normal conditions, IL-1 is expressed at very low levels. Production of IL-1 is regulated at multiple stages: transcription, translation, cleavage of its pro-forms, and release (Vitkovic et al. 2000; Allan et al. 2005; Sims & Smith 2010) (Figure 1.2). Induction of IL-1 transcription occurs in response to a variety of stimuli, including cytokines, products of bacterial infection and necrotic cells, and hypoxia (Hsu & Wen 2002; Perregaux et al. 2002; Allan et al. 2005). IL-1 is translated as pro-forms, and mature IL-1α and β are produced by the cleavage of pro-IL-1α and pro-IL-1β by calpain and caspase-1 respectively. IL-1α and β act via binding to the IL-1 receptor type 1 (IL-1R1); subsequent recruitment of the IL-1 receptor accessory protein (IL-1RACp) triggers intracellular signalling, resulting in activation of the nuclear factor-kB (NF-kB) transcription factor and mitogen-activated protein kinases (MAPKs) (Parnet et al. 2002; Allan et al. 2005). As a consequence, many downstream pro-inflammatory proteins are upregulated, notably including cytokines (IL-6, TNFα), chemokines (CXCL1, CX3CL1) and adhesion molecules (E-selectin, ICAM-1) (Subramaniam et al. 2004; Allan et al. 2005).

Following release, two further mechanisms regulate IL-1’s action: IL-1 receptor antagonist (IL-1Ra) is a naturally occurring antagonist that binds to the IL-1 receptor (IL-1R1), thus preventing agonist action by IL-1. In addition to IL-1R1, another receptor, IL-1R2, exists. This has a much shorter cytoplasmic domain that does not initiate downstream signalling, functioning as a decoy receptor (Colotta et al. 1993).
addition, soluble IL-1 receptors bind secreted IL-1 and prevent it from acting on cells (Allan *et al.* 2005).

**Figure 1.2 - Regulation of IL-1 synthesis.**
Activation of TLR signalling by a PAMP or DAMP induces expression of pro-IL-1α and pro-IL-1β, often referred to as priming. Pro-IL-1α is active and may be released uncleaved from necrotic cells, as an inflammatory stimulus. Cleavage to its mature form is performed by calpain. Pro-IL-1β requires cleavage by caspase-1. Caspase-1 is itself activated via recruitment to an inflammasome. Depicted is the NALP3 inflammasome complex comprising NLRP3 (which detects PAMPs/DAMPs), ASC (a caspase recruitment domain) and pro-caspase-1. Following cleavage, mature IL-1β is secreted. Adapted from Brough *et al.* (2011).

IL-1 exerts effects on many cell types within the CNS, and has multiple downstream processes even within each cell type. For this reason it is difficult to isolate a single mechanism for IL-1’s contribution to neuronal injury. Consistent with its original name of endogenous pyrogen, IL-1 raises body temperature (Cartmell *et al.* 2001). Increased temperature is clinically correlated with a worse outcome in cerebral ischaemia, and IL-1 was thought to contribute to this (Busto *et al.* 1987; Azzimondi *et al.* 1995; Allan...
et al. 2005); however, more recent studies in rats suggest that IL-1’s effects are independent of a pyrogenic response (Spencer et al. 2007; Parry-Jones et al. 2008). Astrocytes are the most prevalent cell type in the CNS, and IL-1 has a profound effect on their number and function. IL-1 induces astrogliosis, and the upregulation in these cells of over 1,000 genes, including both pro- and anti-inflammatory mediators (Allan et al. 2005; John et al. 2005). There exists, therefore, a complicated network of responses downstream of IL-1 binding; the balance between pro- and anti-inflammatory mediators mediates the overall effect of IL-1. IL-1 directly binds to microglia, and can induce release of downstream pro-inflammatory mediators, including chemokines, ROS and prostaglandins, and anti-inflammatory compounds (Aloisi 2001; Basu et al. 2004; Allan et al. 2005). The expression density of IL-1R1 affects how significantly IL-1 affects these cells (Pinteaux et al. 2002), and again, it is postulated that the balance of pro- and anti-inflammatory mediators induced by IL-1 is key to its role in brain injury.

Cerebrovascular endothelial cells express IL-1R1 (Konsman et al. 2004). The action of IL-1 on brain endothelium includes several effects that increase recruitment of immune cells across the BBB into the parenchyma, including expression of cellular adhesion molecules (CAMs), release of chemokines, and junctional BBB breakdown (Bernardes-Silva et al. 2001; Proescholdt et al. 2002; Argaw et al. 2006). Peripheral administration of IL-1β has been demonstrated to induce BBB tight junction protein disruption, facilitating increased neutrophil influx into the brain parenchyma (McColl et al. 2008). In vitro, IL-1α has been demonstrated to mediate the inflammation of mouse brain endothelial cells (MBECs) (Thornton et al. 2010), the cytokine induced cerebrovascular endothelial expression of CAMs, and the release of CXCL1. Specifically, the authors demonstrated that it was IL-1α derived from platelets that drove cerebrovascular inflammation, suggesting an important inflammatory role for platelets in CNS inflammation.

1.6 Platelets in inflammation

1.6.1 Platelets

Platelets are small, non-nucleated cells found in the circulation at high numbers of 150-400 x 10⁹ per litre in humans and 900-1,600 x 10⁹ per litre in mice. The average
adult human therefore has over a trillion platelets in circulation at any one time, and has to synthesise over 100 billion daily due to their short lifespan of just 8-10 days (Semple et al. 2011). Platelets are derived from megakaryocytes in the bone marrow, and play a role in haemostasis, repair and inflammation (Davi & Patrono 2007). Resting platelets resemble smooth discs. Upon activation, their structure transforms to spiny spheres due to calcium mediated changes in their cytoskeleton (Hartwig 1992).

Platelets are activated following vascular injury, as they interact with components of the extracellular matrix (ECM) that are exposed following vascular damage involving denudation of the endothelium. Initial tethering is facilitated by the platelet GpIb/V/IX complex (Mannucci 2004; Davi & Patrono 2007). In particular, GpIb binds to von Willebrand Factor (vWF), a component of the ECM bound to the collagen that forms the basement membrane (Rosenblum 1997). Following initial adhesion, platelets become activated via a network of autocrine and paracrine mediators. Thromboxane A₂ (TXA₂) is released by platelets and binds its own receptor. Downstream signalling then facilitates a rise in intracellular calcium and phosphokinase C, which mediate platelet activation (Davi & Patrono 2007). Platelets and red blood cells release ADP at sites of injury, which binds to P2Y receptors expressed by platelets. Binding to P2Y₁ increases intracellular calcium, and binding to P2Y₁₂ decreases intracellular cyclic AMP (cAMP) (Hollopeter et al. 2001). The final major platelet agonist is thrombin, a protease in the clotting cascade, that binds to protease-activated receptors (PARs) on platelets, which again increase intracellular calcium and decrease cAMP (Davi & Patrono 2007). These three paracrine mechanisms facilitate the recruitment and activation of further platelets at the site of injury. The activation pathways converge on the expression of platelet GpIIb/IIIa, an integrin that is the principle platelet adhesion molecule (Kulkarni et al. 2000).

1.6.2 Clinical anti-platelet agents
Given their role in thrombosis, platelets are implicated in a number of clinical conditions in which atherothrombosis plays a role, such as ischaemic heart disease and stroke. Consequently, a number of anti-platelet medications are used in clinical practice for these conditions. Aspirin belongs to the class of non-steroidal anti-inflammatory
drugs (NSAIDs), whose mechanism of action involves inhibition of the cyclic-oxygenase (COX) enzymes, of which there are at least two: COX-1 and COX-2. Aspirin is a non-selective COX inhibitor, whose anti-platelet effects are mediated through inhibition of the production of TXA\(_2\) by COX in platelets (Davi & Patrono 2007). The fact that platelets are anucleate means they are unable to replace inactivated COX following depletion by aspirin. An important side-effect of NSAIDs, including aspirin, is increased incidence of gastrointestinal bleeding, which is thought to be mediated by COX-1 (Burmester \textit{et al.} 2011). There was interest, therefore, in developing selective COX-2 inhibitors, which would give therapeutic benefit without the side-effect of increased gastrointestinal bleeding (Bombardier \textit{et al.} 2000). Rofecoxib (trade name Vioxx) was one such drug that reached clinical practice, but was recently withdrawn because it increased the incidence of myocardial infarction (Jaeschke \textit{et al.} 2002). Aspirin remains the NSAID of choice for platelet inhibition (Royal Pharmacological Society of Great Britain & British Medical Association 2012). Another class of anti-platelet drugs are the thienopyridines, which includes clopidogrel. These drugs act by irreversible inhibition of P2Y receptors (Hollopeter \textit{et al.} 2001). The latest class of drugs to target platelet function act by antagonising GpIIb/IIIa (Seligsohn 2002). Abciximab is a monoclonal antibody to GpIIb/IIIa, and tirofiban and eptifibatide are small molecule antagonists of the same integrin.

\subsection{1.6.3 Platelets in inflammation}

The classical role for platelets is in blood clotting; however, there is increasing evidence of a complex immunological role for platelets, in both innate (including neutrophil migration) and adaptive responses, as they are capable of expressing CAMs, and releasing cytokines and chemokines (Diacovo \textit{et al.} 1996a; Semple \textit{et al.} 2011).

Platelets store a variety of molecular mediators of inflammation in granules. They have three types of granule: \(\alpha\)-granules, dense granules and lysosomes. The best characterised of these are the \(\alpha\)-granules, which contain CAMs, chemokines (notably CXCL1 and CCL5) and adhesion molecules (Semple \textit{et al.} 2011). Recent evidence has demonstrated that these granules are heterogenous in their contents, and that there may be molecular orchestration of which subtypes are released according to context (Sehgal
While these studies have not investigated differential release of granules in the context of inflammation, it has led to speculation that platelets may have distinct pro- and anti-inflammatory α-granules (Semple et al. 2011). Another secretory body of platelets is the microparticle (MP), a vesicle with a diameter of 200-800nm, which were described over 40 years ago (Wolf 1967; Thiagarajan & Tait 1991). Modern immunological analysis revealed that released MPs, like platelet granules, are heterogenous, and contain different proteins according to what inflammatory stimulus induces their release (Perez-Pujol et al. 2007).

In addition to granules containing pre-synthesised mediators, anucleate platelets contain stable mRNA from which they synthesise proteins de novo (Wicki et al. 1989; Lindemann & Gawaz 2007). A proteomic analysis of the platelet-derived medium revealed that, following platelet activation with thrombin, platelets synthesised a number of immune related proteins including IL-1, TLRs and CD154 (also known as CD40 ligand). Following detection of TLR expression on the surface of platelets (Shiraki et al. 2004; Andonegui et al. 2005), it was later discovered that LPS could directly stimulate translation of IL-1β mRNA to protein within platelets (Shashkin et al. 2008). MPs, in the context of the synovial fluid of rheumatoid arthritis patients, were found to contain both IL-1 ligands, with a 40 fold predominance of IL-1α (Boilard et al. 2010).

Once activated, platelets may secrete IL-1, which can activate various types of peripheral endothelial cells in vitro (Hawrylowicz et al. 1991; Kaplanski et al. 1994; Zarbock et al. 2007; Thornton et al. 2010). Platelets also exhibit membrane-bound IL-1 activity (Lindemann et al. 2001). Platelets stimulate secretion of IL-6 and chemokines CXCL8 and CCL2 from peripheral endothelial cells via an IL-1 dependent mechanism (Kaplanski et al. 1994; Gawaz et al. 2000). One study demonstrated differential secretion of IL-6 and CCL2 between strains of endothelial cells in response to platelet-derived IL-1 (Hawrylowicz et al. 1991).

Platelet accumulation is associated with areas of endothelial activation, and platelets are thought to activate vascular endothelium via IL-1α, IL-1β and CD154 (Hawrylowicz et
al. 1991; Kaplanski et al. 1994; Gawaz et al. 1998; Henn et al. 1998; Gawaz et al. 2000). In addition to chemokine release, platelet-derived IL-1 induces expression of CAMs. In peripheral vascular endothelium IL-1β increased expression of E-selectin, VCAM-1, ICAM-1 and α,β3 integrin, known mediators of neutrophil transmigration (Gawaz et al. 2000). Platelets have been found to support neutrophil migration across peripheral vascular endothelium (Diacovo et al. 1996b; Wegmann et al. 2006; Thornton et al. 2010). They may support different stages of neutrophil recruitment, given that activated platelets strongly enhance the adhesion of neutrophils to endothelium (Zwaginga et al. 1999).

In a study by Thornton et al. (2010), platelets were shown to be key activators of MBECs (Figure 1.3). Activated platelets induced MBEC expression of cellular adhesion molecules (ICAM-1 and VCAM-1) and secretion of CXCL1, and promoted the migration of neutrophils across cultured MBEC monolayers (Thornton et al. 2010). Conditioned medium from platelets drove cerebrovascular endothelial inflammation, an effect blocked by anti-IL-1α antibody, but not anti-IL-1β; and supported transmigration of neutrophils, an effect blocked by IL-1Ra (Thornton et al. 2010). The data supported a role for platelet-derived IL-1α as a key driver of neutrophil migration in the context of cerebrovascular inflammation (Figure 1.3). Clearly, there is a link between platelet function and leukocytes in cerebral inflammation.

1.7 Leukocytes in inflammation

Phagocytes form the main cellular component of the innate immune system, and comprise neutrophils, monocytes and macrophages. These cells originate in the bone marrow and derive from a common progenitor (Akashi et al. 2000). The two classes of phagocyte are monocytes and granulocytes. Monocytes are mononuclear phagocytes that circulate in the bloodstream, while macrophages are more specialised tissue-bound mononuclear cells that may inhabit specific tissues, including microglia in the brain and Kupffer cells in the liver. Monocytes have a role in the phagocytosis of pathogens and cellular debris, the recognition and presentation of antigens, and also play a part in
Platelet IL-1\(\alpha\) drives cerebrovascular inflammation.

Model proposed by Thornton et al. (2010) Platelet-derived IL-1\(\alpha\) induces expression of cellular adhesion molecules (ICAM-1 and VCAM-1) and secretion of chemokine ligand 1 (CXCL1). Platelet IL-1\(\alpha\) supports the transmigration of neutrophils across cerebrovascular endothelium, leading to brain injury.

immunomodulation (Dale et al. 2008). Microglia are the innate immune cells of the brain, thought to derive from pre-haematopoetic myeloid progenitors in the yolk sac (Ginhoux et al. 2010). Perivascular macrophages also inhabit the brain parenchyma (Iadecola & Anrather 2011). Both cell types have an important pro-inflammatory role, being capable of producing cytokines, such as IL-1\(\beta\), TNF\(\alpha\) and ROS.

Polymorphonuclear granulocytes (PMNs) are another class of phagocyte, over 90% of which are neutrophils (Dale et al. 2008). While the neutrophil is classically considered a phagocyte for clearing pathogens, more recent evidence has demonstrated they secrete cytokines, form extracellular traps, and have a regulatory role in both innate and adaptive immunity (Brinkmann et al. 2004; Mantovani et al. 2011).
During an inflammatory response, neutrophils quickly migrate into affected tissue, where they inactivate, bind and phagocytose pathogens or necrotic cells. Inactivation of phagocytosed material occurs via proteolytic enzymes, ROS and antimicrobial proteins (Elsback & Weiss 1992; Klebanoff 1999). In addition, neutrophils exert extracellular effects: via degranulation and the release of cytokines and ROS (Lacy 2006). More recent evidence has demonstrated that neutrophils generate fibres, which form neutrophil extracellular traps (NETs), which inactivate bacteria (Brinkmann et al. 2004). Recent evidence has uncovered the process by which neutrophils migrate into inflamed tissue.

1.8 Transendothelial migration of neutrophils

The migration of neutrophils across vascular endothelium has classically been described in four phases: rolling, activation, arrest and transmigration (Wagner & Roth 1999). More recent work on the molecular mechanisms of transmigration has refined the model to include six stages: capture, slow rolling, adhesion strengthening, intraluminal crawling, paracellular or transcellular migration, and basement membrane migration (Ley et al. 2007) (Figure 1.4). These stages are supported by different CAMs. Recruitment of neutrophils and trafficking of monocytes involves their adhesion to and transmigration across the walls of blood vessels. CAMs facilitate these processes in what is now conceptualised as an adhesion cascade (Ley et al. 2007). There are five classes of CAM: cadherins, the immunoglobulin (Ig) superfamily, selectins, mucins, and integrins (Lodish et al. 2000).

The capture phase is characterised by initial loose binding between neutrophils and endothelial cells. This occurs between the selectin class of adhesion molecules (including L-, E-, and P-selectin) and sugar groups on selectin ligand molecules, notably including P-selectin glycoprotein ligand-1 (PSGL1) (McEver & Cummings 1997; Wagner & Roth 1999; Ley et al. 2007). L-selectin predominates on the surface of leukocytes, while E- and P-selectin are upregulated by inflamed endothelial cells. PSGL1 is expressed both by leukocytes and the endothelium. Direct binding occurs between neutrophils and endothelial cells, and secondary leukocyte-leukocyte capture via L-selectin also contributes to neutrophil recruitment (Eriksson et al. 2001). Rolling
occurs due to the removal of loosely bound neutrophils, induced by the shear stress of
blood flow, and subsequent rebinding. The bond that forms between P-selectin and
PSGL1 exhibits high tensile strength and fast capture/dissociation (on/off) rates (Alon et
al. 1995), which allows neutrophil capture via one or few bonds; indeed, capture via
selectins actually requires shear stress for bonds to form (Finger et al. 1996; Ley et al.
2007). It is postulated that this prevents inappropriate neutrophil capture in static or
slow moving blood. Selectin binding induces neutrophil activation; while the
mechanisms are not fully elucidated, there is evidence that binding to E-selectin triggers
signal transduction in neutrophils leading to the expression of integrins, which facilitate
adhesion strengthening (Simon et al. 2000). In addition, integrin expression on
neutrophils is initiated via intracellular signalling downstream of bound L-selectin
(Williams & Solomkin 1999).
Figure 1.4 – Neutrophil migration across the vascular endothelium.
Transendothelial migration is now understood to comprise seven steps: capture, rolling, slow rolling, arrest, spreading, crawling and migration via a paracellular or transcellular route. Here, each stage is pictured above the molecular mediators that facilitate neutrophil-endothelial interaction. Adapted from Ley et al. 2007.
Adhesion strengthening of neutrophils to vascular endothelium occurs through interaction of integrins on neutrophils with CAMs expressed by the endothelium. Initiation of this step is facilitated by inflammatory activation of the epithelium to express adhesion molecules including ICAM-1, ICAM-2 and VCAM-1 (Hashimoto et al. 1994); and by chemokine action on neutrophils, stimulating expression of activated integrins, particularly $\beta_1$ and $\beta_2$ integrins (Campbell et al. 1996; 1998; Murphy 2008). Chemokines also increase the affinity of integrin binding (Constantin et al. 2000; Shamri et al. 2005), leading eventually to arrest (Chigaev et al. 2003). Specificity in the site of arrest is achieved through complex networks of chemokine-induced integrin signalling, involving over 900 proteins and 6,000 interactions (D'Ambrosio et al. 2002; Ley et al. 2007).

Following their arrest on the endothelium, neutrophils must migrate across the endothelial layer, pass through the basement membrane. Transendothelial migration may occur via paracellular (between the cells) or transcellular (through the cells) routes. Following arrest, neutrophils crawl along the endothelium towards optimal migration sites in a process dependent on Mac-1 binding (Phillipson et al. 2006). Such sites may be endothelial tight junctions (paracellular) or thinner endothelial cells (transcellular). In addition, PECAM1, expressed on neutrophils and endothelial cells and that binds itself (Vaporciyan et al. 1993; Newman 1997), is concentrated at intercellular junctions on the endothelium (Newman 1997). It therefore acts as a ‘homing receptor’ for migrating neutrophils. Paracellular migration is facilitated by binding via junctional adhesion molecules (JAMs), PECAM1, ICAM-1 and CD99 (Schenkel et al. 2002; Lou et al. 2007; Ley et al. 2007).

Interestingly, CD99 blockade inhibits paracellular migration at a site downstream of PECAM binding, and blockade of both has additive effects, suggesting sequential steps in migration via tight junctions (Lou et al. 2007). Transcellular migration has been observed in vivo (Feng et al. 1998), has been demonstrated to be responsible for up to 20% of neutrophil migration in vitro (Carman & Springer 2004), and may occur via vesiculo-vacuolar organelles (VVOs) that form channel like structure through which the neutrophils pass (Dvorak & Feng 2001). While the exact mechanism remains elusive,
transcellular migration is thought to be dependent on binding via PECAM1 and other CAMs involved in paracellular migration (Engelhardt & Wolburg 2004; Ley et al. 2007).

Once through the endothelial cell layer, migrating neutrophils must penetrate the perivascular basement membrane, with its associated pericytes. The basement membrane is a protein-matrix layer, primarily comprising laminin and collagen type IV (Wang et al. 2005). Confocal microscopy has revealed areas of low expression of these key components, and that these areas co-localised with gaps in pericyte end feet (Wang et al. 2006). Such sites were enlarged by stimulation with the pro-inflammatory IL-1ß, and were preferentially used by migrating neutrophils (Wang et al. 2006). In addition, there is evidence that migrating neutrophils express proteases (notably elastase) on their surface, which both breaks down the basement membrane layer, and exposes new leukocyte-matrix interaction sites (Wang et al. 2005; Ley et al. 2007).

1.9 Cerebrovascular inflammation in clinical conditions
Cerebrovascular inflammation is an important contributor to neurological conditions, including stroke, multiple sclerosis, epilepsy and Alzheimer’s disease (Allan et al. 2005). Together, these conditions exert an enormous clinical burden; cerebral ischaemia alone affects 15 million people each year, 6 million of whom do not survive (World Stroke Organisation 2012). Those who do are left with significant morbidity, dependent on others for everyday activity.

IL-1 is implicated in a variety of CNS conditions of different aetiology. IL-1 plays an important role in the acute insults involved in epilepsy and stroke (Allan et al. 2005). IL-1 is upregulated in the CNS in response to a seizure (Rijkers et al. 2009), though it is not clear whether this is a response to the seizure or to the resulting neuronal injury. There is evidence that anti-IL-1 therapy may lower seizure threshold, which has led to suggestions that such a strategy may lead to the development of anti-convulsive medication (De Simoni et al. 2000; Vezzani et al. 2000; Rijkers et al. 2009).
Within the context of cerebral ischaemia, IL-1 is upregulated at the mRNA level within minutes of onset, and is detectable as a protein within hours (Boutin et al. 2003; Allan et al. 2005). There is much evidence for the neuroprotective effect of anti-IL-1 interventions, including recombinant IL-1Ra (Relton & Rothwell 1992; Pradillo et al. 2012), neutralising antibody (Yamasaki et al. 1995), inhibition of protein cleavage (Hara et al. 1997), and genetic knockout (Boutin et al. 2001). Interestingly, neuroprotection in experimental stroke required the genetic knockout of both IL-1 ligands; single knockouts of either IL-1α or β failed to affect brain injury, an observation attributed to compensation in IL-1α knockout mice and IL-1 independent pathways in IL-1β knockouts (Boutin et al. 2001).

Given the diverse mechanisms by which IL-1 has the potential to exacerbate brain injury, there is much interest in targeting its effects therapeutically. Examples of the strategies in development for cerebral ischaemia are shown in Table 1.2. Recombinant IL-1Ra is the most advanced of these therapies. IL-1Ra has demonstrated neuroprotection in vivo in cerebral ischaemia (Allan et al. 2005; Pradillo et al. 2012), and has met targets in early clinical trials (Emsley et al. 2005; Clark et al. 2008; Galea et al. 2011); however, the majority of drugs in development target the IL-1β ligand, and demonstration of the role of IL-1α in mediating cerebrovascular inflammation, may lead to novel therapeutic strategies.

Following the onset of CNS inflammation, immune cells migrate to and accumulate in the brain parenchyma. Neutrophils are implicated in a number of CNS diseases. They play an important role in combating the infection in bacterial meningitis (Koedel et al. 2010). The role of neutrophils in MS, a sterile condition, remains unclear: while neutrophils have not been detected in human MS lesions post-mortem (Holman et al. 2011), they are implicated in neuromyelitis optica, another CNS autoimmune pathology (Wingerchuk et al. 2007), and in animal models of MS (McColl et al. 1998). In experimental stroke, neutrophils appear in cerebral hemispheres as early as 4h after ischaemia onset, overwhelm the tissue by 72h, before declining in number after 7 days; a temporal pattern supporting a causative role in post-ischaemic brain injury (McColl et al. 2007; Gelderblom et al. 2009). There is also evidence from clinical imaging studies.
that neutrophils migrate into the CNS within 24h of stroke onset (Price et al. 2004; Buck et al. 2008). Some studies have contradicted this consensus (Fassbender et al. 2002; Maier et al. 2004). There is significant in vitro evidence that migrated neutrophils rapidly assume a neurotoxic phenotype (Dinkel et al. 2004; Shaw et al. 2008; Allen et al. 2012), while in vivo experiments using knockout mice and other anti-neutrophil interventions have further demonstrated a deleterious role for neutrophils (Chen et al. 1994; Connolly et al. 1996; Dawson et al. 1996; Yenari et al. 1998; Beray-Berthat et al. 2003).

<table>
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<tr>
<th>Drug</th>
<th>Target</th>
<th>IL-1α or β</th>
<th>Mechanism</th>
<th>Trial stage</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1Ra</td>
<td>IL-1R1</td>
<td>Both</td>
<td>Antagonist</td>
<td>Phase II</td>
<td>Emsley et al. (2005)</td>
</tr>
<tr>
<td>Anti-IL-1β antibody</td>
<td>IL-1β</td>
<td>IL-1β</td>
<td>Neutralisation</td>
<td>Pre-clinical</td>
<td>Yamasaki et al. (1995)</td>
</tr>
<tr>
<td>Anti-NLRP1 antibody</td>
<td>NLRP1</td>
<td>IL-1β</td>
<td>Inhibits caspase-1 activation</td>
<td>Pre-clinical</td>
<td>Abulafia et al. (2009)</td>
</tr>
<tr>
<td>Anti-ASC antibody</td>
<td>ASC</td>
<td>IL-1β</td>
<td>Inhibits caspase-1 activation</td>
<td>Pre-clinical</td>
<td>de Rivero Vaccari et al. (2008; 2009)</td>
</tr>
<tr>
<td>Caspase-1 inhibitor</td>
<td>Caspase-1</td>
<td>IL-1β</td>
<td>Inhibits pro-IL-1β cleavage</td>
<td>Pre-clinical</td>
<td>Ross et al. (2007)</td>
</tr>
<tr>
<td>Glyburide</td>
<td>NLRP3</td>
<td>IL-1β</td>
<td>Inhibits caspase-1 activation</td>
<td>Pre-clinical</td>
<td>Suzuki et al. (2009)</td>
</tr>
<tr>
<td>CA074</td>
<td>Cathepsin B</td>
<td>IL-1β</td>
<td>Inhibits caspase-1 activation</td>
<td>Pre-clinical</td>
<td>Benchoua et al. (2004)</td>
</tr>
<tr>
<td>B27-HYD</td>
<td>Calpain</td>
<td>IL-1α</td>
<td>Inhibits pro-IL-1α cleavage</td>
<td>Pre-clinical</td>
<td>Anagli et al. (2009)</td>
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<tr>
<td>SB 239063</td>
<td>P38 MAPK</td>
<td>Both</td>
<td>Inhibits expression</td>
<td>Pre-clinical</td>
<td>Barone et al. (2001)</td>
</tr>
<tr>
<td>BMS-345541</td>
<td>IKK</td>
<td>Both</td>
<td>Inhibits expression</td>
<td>Pre-clinical</td>
<td>Herman et al. (2005)</td>
</tr>
</tbody>
</table>

Table 1.2 – Anti-IL-1 interventions in development for cerebral ischaemia. A diverse range of therapeutic mechanisms are undergoing development to target IL-1 expression, release and action. The majority target IL-1β. Adapted from Brough et al. (2011).
Platelets have been implicated in the pathogenesis of inflammation in cerebral ischaemia and MS. Activated platelets are adherent to vascular endothelium following stroke in feline and canine models, and are present in human MS lesions (Kochanek et al. 1988; Jafar et al. 1989; Marquardt et al. 2002; Karakantza et al. 2003; Langer et al. 2012). Further, inhibition of platelet function attenuates a mouse model of MS (Langer et al. 2012). Platelets have been most extensively studied in ischaemic stroke, given that clotting is a long-known function of platelets and plays a causative role in thromboembolic stroke. The reduction in blood flow, during a stroke, creates a hypoxic environment, and drastically alters the shear stress on the cerebrovascular endothelium; these changes lead to the activation of the complement cascade and circulating platelets, and to an endothelial cell response (Pinsky et al. 1996; Carden & Granger 2000; Peerschke et al. 2010; Eltzschig & Carmeliet 2011; Iadecola & Anrather 2011). Fibrin production follows, and this leads to further aggregation of platelets and leukocytes (del Zoppo et al. 1991; Hyman et al. 2009). Aggregation creates micro-occlusions, and the establishment of a positive feedback loop driving further inflammation. Adhesion molecules are upregulated and expressed by platelets, leukocytes and the endothelium, and secreted chemokines attract further leukocytes to the ischaemic site (Yilmaz & Granger 2010; Iadecola & Anrather 2011). The BBB’s permeability is increased by numerous factors. Hypoxia induces an increase in the number of leukocyte transport vesicles in endothelial cells promoting transcellular migration (Engelhardt & Sorokin 2009; Iadecola & Anrather 2011). Leukocytes release proteases which break down endothelial junctional adhesion molecules (JAMs); JAMs themselves are downregulated by endothelial cells, thus promoting paracellular migration of neutrophils (Engelhardt & Sorokin 2009; Iadecola & Anrather 2011).
1.10 Summary and aims

Extensive evidence suggests inflammation is an important contributor to neuronal injury and that migration of neutrophils into the brain parenchyma is central to this effect in several conditions. Recent evidence has demonstrated a supportive role for platelets, and specifically platelet-derived IL-1α, in the transmigration of neutrophils across cultured MBECs. This evidence for the role of platelet-derived IL-1α in neutrophil transmigration has been conducted in vitro; the mechanism has not been demonstrated in the more complex inflammatory milieu that exists in vivo.

The overall aim of this thesis was to determine the role of platelet-derived IL-1α in driving neutrophil migration in vivo. To address this, specific objectives were to:

1. Characterise in vivo models of neutrophil migration.
2. Determine the effects of platelets on neutrophil migration in vivo.
3. Determine the effects of IL-1 on neutrophil migration in vivo.
4. Determine the effects of platelet-derived IL-1α on neutrophil migration in vivo.
Chapter 2

General materials and methods
2.1 Animals
Experiments were performed on male inbred C57BL/6J mice (Harlan Laboratories, Bicester, UK) weighing approximately 25g except where stated otherwise. Genetic knockout mice were bred in house. Mice were housed under controlled conditions of temperature and humidity in individually-ventilated cages. Mice were fed ad libitum with standard, pelleted rodent chow and had free access to water. Mice were housed in a 12h light-dark cycle. The experimental protocol was performed under United Kingdom Home Office personal and project licences, using protocols which adhered to the UK Animals (Scientific Procedures) Act 1986.

2.2 Animal models
2.2.1 Peritoneal inflammation model
Mice were briefly anaesthetised with isoflurane (3%; Baxter, Newbury, UK) in O2 (200ml/min) and N2O (400ml/min) and injected intraperitoneally with 1mg/kg LPS from Escherichia coli O127:B8 (Sigma-Aldrich, Dorset, UK) or vehicle (PBS) in a volume of 8ml/kg. Mice were subsequently recovered. At indicated time points post-injection, mice were re-anaesthetised as above and killed via neck dislocation. Peritoneal lavage was performed using 5ml PBS containing 0.1% BSA and 1mM EDTA, injected into the peritoneal cavity. The peritoneal wall was massaged and the fluid removed, as described previously (Wegmann et al. 2006; Borges et al. 1997; Bosse & Vestweber 1994).

2.2.2 Air pouch inflammation model
Dorsal air pouches were created in conscious mice by subcutaneous injection of sterile-filtered air. Creation and maintenance of air pouches took place over seven days to allow any inflammation induced by the initial injection to resolve. At day 0, 4ml air was administered to create the pouch, and at day 4, 3ml air was administered for pouch maintenance as described previously (Kukulski et al. 2007). At day 7, 1ml of LPS from Escherichia coli O127:B8 (1mg/ml) or vehicle (PBS) was injected into the air pouch. Mice were killed via neck dislocation, under gaseous anaesthesia as in 2.2.1, at indicated time points post-injection. Air pouch lavage was performed using 4ml PBS,
with 0.1% BSA and 1mM EDTA, injected into the air pouch. The air pouch was massaged and the fluid removed.

2.2.3 Broncho-alveolar inflammation model
Mice were exposed to aerosolised LPS from *Escherichia coli* O127:B8 (2mg/ml, Sigma-Aldrich, Dorset, UK) or vehicle (saline) for 20 min via a nebuliser chamber. Mice were killed via neck dislocation under anaesthesia 6h after exposure. Broncho-alveolar lavage was performed, via direct cannulation of the trachea, with 1ml of PBS containing 0.1% BSA and 1mM EDTA.

2.2.4 Cerebral inflammation model
Animals were anaesthetised using 2–3% isoflurane in O₂ (200ml/min) and N₂O (400ml/min) and then placed securely in a small animal stereotaxic frame (Stoetling, Illinois, USA). Anaesthesia was maintained via a face mask in a non-ventilated animal. Depth of anaesthesia was monitored throughout the procedure by respiratory rate and toe pinch reflex. Core body temperature was maintained at 37°C using a rectal sensor and thermal mat (Harvard Apparatus, Tonbridge, UK). The skin overlying the skull was shaved and cleaned with Videne® (Adams Healthcare, Leeds, UK). The skull was exposed via a midline incision and a small-hole craniotomy performed. Mice were injected intracerebrally with 1µl LPS from *Escherichia coli* O127:B8 (4mg/ml, Sigma-Aldrich, Dorset, UK), via a glass micro-needle. The LPS solution was lightly coloured with a small amount of monastral blue dye (to visualise the solution in the micro-needle and locate the injection site when sectioning tissue). The glass micro-needle was made by pulling glass micro-capillary pipettes with calibration marks at every 1µl (Drummond Scientific Company, Broomall, Pennsylvania, USA) on a vertical electrode puller (Model PP830, Narishige, Japan) as described previously (McCluskey *et al.* 2008). The micro-needle was connected by tubing to an empty 10 ml syringe, which was used to infuse the solution. Co-ordinates for injection from bregma: anterior-posterior -0.0mm, lateral -2.0mm, deep -2.5mm. Injections were carried out at a rate of 0.5µl/min. The micro-needle was left *in situ* for 2 min following the injection. Following removal of the needle, the wound was closed with 2-3 interrupted sutures (Ethicon, Livingston, UK), and re-cleaned with Videne. EMLA cream containing lidocaine and prilocaine
AstraZeneca, London, UK) was applied to the wound. O₂ (500ml/min) was administered until recovery of consciousness. Surgery was performed under aseptic conditions, with sterile instruments, reagents and theatre dress.

Post-operatively, animals were re-housed individually for 2h while recovery was monitored. Cages were placed on a heated mat, and pelleted food and water were supplemented with moist, mashed food. After 2h, animals were re-housed in cages of five, to prevent social isolation from affecting neuroinflammatory responses as has been previously reported (Karelina et al. 2011). Recovery monitoring, in addition to the usual checks by the animal staff, continued until sacrifice at a maximum of 24h post-operatively.

2.2.5 Blood sampling
Cardiac blood sampling was performed under anaesthesia immediately prior to sacrifice. Cardiac puncture was performed using a 0.5ml insulin syringe containing 50µl of 3.8% sodium citrate solution to prevent coagulation. Puncture was performed through the chest wall, or through direct visualisation of the left ventricle, where the mice were to undergo transcardiac perfusion. Approximately 500µl of blood was sampled from each animal.

Blood samples without sacrifice were taken via the tail vein. A scalpel blade was used to puncture the vein, and 20-50µl of blood was collected into an EDTA-coated tube (International Scientific Supplies, Bradford, UK). A gauze was applied under pressure to the tail wound to stop bleeding.

2.2.6 Transcardiac perfusion
Transcardiac perfusion with saline was undertaken to remove blood from tissue samples prior to processing or fixation. At the relevant time point, mice were anaesthetised with isoflurane (3%) in O₂ (200ml/min) and N₂O (400ml/min). The chest cavity was opened surgically, to allow direct visualisation of the heart. Up to 500µl blood was sampled via cardiac puncture as described above. A 21G butterfly needle was inserted and secured in the left ventricle. The right atrium was punctured to create an outflow from the
circulation. Animals were perfused transcardially with 20ml ice-cold 0.9% saline over 2 min. Where paraformaldehyde fixation was not required, tissue samples were removed and stored on dry ice, prior to longer term storage at -80°C.

2.2.7 Fixation by paraformaldehyde
Paraformaldehyde (PFA) may be used as a fixative. Its mechanism of action involves denaturation of proteases and the formation of covalent bonds between parenchymal proteins. PFA fixation was undertaken via transcardiac perfusion immediately following perfusion with saline. 4% PFA was prepared in phosphate buffer (PB), filtered and adjusted to pH 7.4. 20ml 4% ice-cold PFA was transcardially perfused over 2 min. Following perfusion, tissue samples were harvested and post-fixed for 24h in 4% PFA followed by 24h in 20% cryoprotectant (sucrose in PB) solution. Samples subsequently underwent snap-freezing in isopentane on dry ice and were stored at -20°C.

2.3 Preparation and analysis of brain tissue
2.3.1 Brain sectioning
Brains were removed from frozen storage and mounted in distilled water on a frozen sledge microtome slicing chuck (Bright Instruments, Huntingdon, UK). The entire brain was cut into serial 20µm coronal sections, at 240µm intervals. Sections were stored in cryoprotectant antifreeze at -20°C, as described previously (Hoffman & Wei Wei Le 2004).

2.3.2 Immunohistochemistry - single antibody labelling
Immunohistochemistry (IHC) uses antibodies to detect target antigens in tissue sections. Here, IHC was used to detect specific proteins and cellular antigens. Brain sections, stored in cryoprotectant, were washed in PBS and incubated for 30 min in 0.3% H₂O₂ in dH₂O to block endogenous peroxidase activity. After further PBS wash steps, sections were incubated in 10% normal serum (Vector Laboratories, Peterborough, UK) in PBS with 0.5% BSA. The species of normal serum used was the same as the species which raised secondary antibody was raised in. Sections were washed in PBS again, and incubated overnight at 4°C in primary antibody against the desired protein. Sections were washed the next day and incubated in 0.5% biotinylated secondary antibodies in
PBS for 1h, followed by washing and incubation with avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories, Peterborough, UK) for 1h. Fuller details of biological reagents used are in Table 2.1. Diaminobenzidine (DAB) was added for 2-5 min; a substrate for peroxidase, DAB undergoes a conversion to a brown visible colour, which can be quantified. Nickel-enhanced DAB staining was undertaken also. Following addition of ABC kit, sections were washed in 0.1M sodium acetate, and developed with a solution of DAB and 1.5% ammonium nickel (ii) sulphate hexahydrate contained in 0.1M sodium acetate. This reaction was stopped by washing with 0.1M sodium acetate.

Sections were mounted on gelatine-coated microscope slides and dehydrated through serial concentrations of ethanol: 70% for 2 min, 90% for 2 min, 100% for 5 min and xylene for 10 min. Cover slips were subsequently applied with DePex medium.

<table>
<thead>
<tr>
<th>Target</th>
<th>1° Ab</th>
<th>Supplier</th>
<th>Species</th>
<th>Concentration</th>
<th>2° Ab</th>
<th>Normal serum species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>SJC4</td>
<td>SJ Campbell, Oxford, UK</td>
<td>Rabbit</td>
<td>1:50,000</td>
<td>Goat anti-rabbit</td>
<td>Goat</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Goat anti-mouse</td>
<td>Goat</td>
</tr>
<tr>
<td>Rat IgG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Goat anti-rat</td>
<td>Goat</td>
</tr>
</tbody>
</table>

Table 2.1 - Antibodies used for DAB immunohistochemistry

2.3.3 Immunohistochemistry - fluorescent double antibody labelling

Fluorescent double labelling enables visualisation of co-localised antigens in a single section. Brain sections, stored in cryoprotectant, were washed in PBS and incubated in 10% normal serum (Vector Laboratories, Peterborough, UK) in reagent diluent (RD; PBS with 0.5% BSA). Sections were then incubated overnight in primary antibody against the desired protein. Sections were washed the next day and incubated in fluorophore-conjugated secondary antibody in primary diluent (see Appendix 7.1) for 3h, followed by washing in PBS. Fuller details of biological reagents used are found in Table 2.2. Sections were then mounted on gelatine-coated microscope slides and coverslipped with ProLong Gold mounting medium (inVitrogen, Paisley, UK).
<table>
<thead>
<tr>
<th>Target</th>
<th>1° Ab</th>
<th>Supplier</th>
<th>Species</th>
<th>Conc</th>
<th>2° Ab</th>
<th>Normal serum species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>CD45</td>
<td>AbCam</td>
<td>Rat</td>
<td>1:200</td>
<td>Donkey anti-rat</td>
<td>Donkey</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>SJC4</td>
<td>SJ Campbell, Oxford, UK</td>
<td>Rabbit</td>
<td>1:10,000</td>
<td>Donkey anti-rabbit</td>
<td>Donkey</td>
</tr>
<tr>
<td>IL-1α</td>
<td>IL-1α</td>
<td>R&amp;D</td>
<td>Goat</td>
<td>1:100</td>
<td>Donkey anti-rat</td>
<td>Donkey</td>
</tr>
<tr>
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<td>Iba1</td>
<td>Wako</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Donkey anti-rabbit</td>
<td>Donkey</td>
</tr>
<tr>
<td>Platelets</td>
<td>CD41</td>
<td>BD Bioscience</td>
<td>Rat</td>
<td>1:100</td>
<td>Donkey anti-rat</td>
<td>Donkey</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>VCAM-1</td>
<td>R&amp;D</td>
<td>Goat</td>
<td>1:250</td>
<td>Donkey anti-goat</td>
<td>Donkey</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>IL-1Ra</td>
<td>R&amp;D</td>
<td>Goat</td>
<td>1:500</td>
<td>Donkey anti-goat</td>
<td>Donkey</td>
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</tbody>
</table>

Table 2.2 - Antibodies used for immunofluorescence

2.3.4 Microscopy

Bright-field images were collected on a Axioskop upright microscope (Carl Zeiss, Cambridge, UK) using 5x/0.25, 10x/0.50 Fluar and 20x/0.75, 40x/0.95 Plan Apochromat objectives and captured using a Axiocam colour CCD camera (Carl Zeiss, Cambridge, UK) through Axiovision Software (Carl Zeiss, Cambridge, UK).

Fluorescence images were collected on a Olympus BX51 upright microscope (Olympus, Southend, UK) using 4x/0.13, 10x/0.30, 20x/0.50, 40x/0.75 Plan Fln objectives and captured using a Coolsnap ES camera (Photometrics, Tucson, USA) through MetaVue Software (Molecular Devices, Sunnyvale, USA). Specific band pass filter sets for DAPI, FITC and Texas Red were used to prevent bleed through from one channel to the next. All images were then processed and analysed using ImageJ (NIH, Bethesda, USA).

2.3.5 Tissue homogenisation

Brains were cut along the midline, and each hemisphere was homogenised in 250μl buffer (see Appendix 7.1) using a hand homogeniser (Kontes, Vineland, USA) and an ultrasonic homogeniser (IKA, Staufen, Germany). Samples were kept at 4°C on ice, and centrifuged at 17,000g for 30 min at 4°C. Supernatants were kept frozen for analysis.
2.3.5 Bicinchoninic acid (BCA) protein assay

A BCA assay (Pierce, Cramlington, UK) was carried out immediately after homogenisation on homogenate supernatant. Serial dilutions of BSA were used as standards. Samples were diluted 1:50 and 50µl added in duplicate to a 96 well plate. BCA reagent was prepared, comprising a 50:1 ratio of reagents A and B, and 200µl added to each well. This was incubated at 37°C for 30 min. Absorbance of the coloured product of the BCA reaction was read at 570nm on a plate reader (MRX, Dynatech, Willenhall, UK), and the protein concentration in samples derived from the standard curve.

2.4 Flow cytometry

Suspended cells are streamlined by a fluidics system into a flow of single cells. Multiple lasers interrogate the cells as they flow past optical detectors. Fluorophore-conjugated antibodies are used to label cells of interest. When labelled cells pass through the laser beam, the fluorochromes are stimulated and emit light signals, which are converted to a digital signature by the optical detectors. Cell size and granularity are quantified respectively by the degree of forward scatter (FSC) and side scatter (SSC) of the laser light (Figure 2.1). Large, granular cells such as neutrophils have a high FSC and SSC.

2.4.1 Acquisition of flow cytometric data

Flow cytometry was used to quantify cell populations in lavage and blood samples. 200µl of lavage fluid underwent analysis. 50µl of blood was added to 50µl buffer (0.1% BSA, 1mM EDTA in PBS). Samples were incubated for 20 min with 1:200 rat anti-mouse CD16/CD32 to block non-specific Fc binding. Cocktails of fluorophore-conjugated antibodies were added for 30 min (Table 2.3). Red blood cells in samples were then lysed by the addition of 450ml FACS Lysing Solution (BD Biosciences, Oxford, UK). Absolute numbers of cells were determined through the use of TruCOUNT™ tubes (BD Biosciences, Oxford, UK), or by the addition of 50µl fluorescent counting beads (inVitrogen, Paisley, UK). Flow cytometry was performed on a CyAn™ ADP Flow Cytometer (Dako UK Ltd, Ely, UK) equipped with 405, 488 and 633nm lasers using Summit 4.0 software. The following detection filters were fitted: 530/40nm bandpass, 575/25nm bandpass, 613/20nm bandpass, 680/30nm
bandpass, 450/50 bandpass, 530/30 bandpass, 665/20nm bandpass, and 750nm long-pass.

**Figure 2.1 – Principles of flow cytometry.**
(a) Neutrophils are labelled with antibodies to Ly-6G (red) and CD45 (blue). (b) Labelled cells in samples are funnelled into single file, and pass through a laser beam. Forward and side scatter of laser light, and fluorescence from fluorophore-conjugated antibodies are detected by the flow cytometer. (c) Forward scatter denotes cell size, side scatter denotes granularity. Fluorescent emissions denote which antibodies have bound to a cell, and hence its immunophenotype.

**2.4.2 Analysis of flow cytometric data**
Cell populations were determined on Summit 4.0 software via positive labelling of relevant markers. Compensation for crossover between emission signal wavelengths was determined using single-labelled and unlabelled samples. An example gating strategy for neutrophils is described in Appendix 7.2. For blood samples, a minimum of 1000 beads, 1,000 neutrophils or 5,000 leukocytes (whichever threshold occurred last) were acquired per sample. For lavage samples, a minimum of 20,000 cellular events were acquired per sample.
<table>
<thead>
<tr>
<th>Target</th>
<th>Antigen</th>
<th>Fluorochrome</th>
<th>Conc</th>
<th>Supplier</th>
<th>Excitation λ</th>
<th>Emission λ</th>
</tr>
</thead>
<tbody>
<tr>
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<td>eBioscience</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
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<td>PE</td>
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<td>BD Pharmingen</td>
<td>496</td>
<td>578</td>
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<td>APC</td>
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<td>eBioscience</td>
<td>650</td>
<td>660</td>
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<td>Leukocytes</td>
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<td>PerCP-Cy5.5</td>
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<td>eBioscience</td>
<td>482</td>
<td>695</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>MHC II</td>
<td>APC</td>
<td>1:100</td>
<td>eBioscience</td>
<td>650</td>
<td>660</td>
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<tr>
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<td>eBioscience</td>
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<td>695</td>
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<td>519</td>
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<td>FITC</td>
<td>1:100</td>
<td>BD Pharmingen</td>
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<td>519</td>
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<td>1:100</td>
<td>BD Pharmingen</td>
<td>496</td>
<td>578</td>
</tr>
<tr>
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<td>FITC</td>
<td>1:200</td>
<td>BD Pharmingen</td>
<td>494</td>
<td>519</td>
</tr>
<tr>
<td>Platelets</td>
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<td>PE</td>
<td>1:200</td>
<td>BD Pharmingen</td>
<td>496</td>
<td>578</td>
</tr>
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<td>FITC</td>
<td>1:100</td>
<td>AbD Serotec</td>
<td>494</td>
<td>519</td>
</tr>
</tbody>
</table>

Table 2.3 - Antibodies used for flow cytometry

2.5 Coulter Counter

20µl of each blood and lavage samples underwent analysis for leukocyte and platelet count, via a Coulter Counter (Beckman Coulter AcT 8 High Wycombe, UK), using the manufacturer’s protocol.

2.6 Quantification of neutrophil accumulation in tissues

The absolute number of neutrophils was determined using three methods. Two approaches were used in lavage fluids. First, the proportion of positively-labelled cells in flow cytometry data was multiplied by total cell count from direct haemocytometry. Second, the proportion of CD45 positive cells that were also Ly-6G positive was multiplied by leukocyte count from Coulter Counter measurements.

In blood samples numbers of neutrophils and total leukocytes were calculated using fluorescent micro-beads, as described previously (Perruche et al. 2004).
Total cell numbers in brain tissue sections were determined via microscopy following immunohistochemistry staining. Anti-neutrophil (SJC4, rabbit anti-mouse) primary antibody (1:50,000; kindly provided by Drs Daniel Anthony and Sandra Campbell, University of Oxford, Oxford, UK) was used to stain for neutrophils. The counting map is shown in Figure 2.2. Neutrophils were quantified using a 10x10mm graticule at 20x magnification, and by a total of the three areas surveyed.

![Figure 2.2 – Neutrophil quantification in coronal brain sections](image)

Schematic showing location of brain areas used for counting neutrophils in coronal brain sections. Squares represent 320µm² at the 20x magnification used for counting.

### 2.7 Protein quantification

#### 2.7.1 Enzyme-linked immunosorbent assay (ELISA)

Cytokine concentrations in lavage fluid were determined by ELISA (Figure 2.3). IL-1α, IL-1β, IL-6, KC (CXCL1) and RANTES (CCL5) in peritoneal lavage fluid were assayed using a validated mouse-specific ELISA DuoSet (R&D Systems, Abingdon, UK). A 96 well plate (Nunc, Roskilde, Denmark) was coated overnight at 4°C with capture antibody (50µl) at concentrations recommended by the manufacturer. Subsequently, non-specific binding was blocked for 1h with 1% BSA (300µl). Samples (50µl) were incubated in each well for 2h at 4°C. Biotinylated detection antibody (50µl), at concentrations recommended by the manufacturer, was added to each well and incubated for 2h at 4°C. Streptavidin horseradish peroxidase (HRP; 50µl), at 1:200 in 1% BSA, was added for 20 min incubation at 4°C. Finally, a 1:1 mixture of H₂O₂ and tetramethylbenzidine was added as substrate solution (R&D Systems, Abingdon, UK) for approximately 20 min, until a visible colour change was seen in the standards, when the reaction was halted via addition of 25µl 1M NH₄SO₄. Each step was punctuated by a
triple plate wash using 0.1% Tween in PBS. Standards were assayed in triplicate, and samples in duplicate. Absorbance was measured using a plate reader (MRX, Dynatech, Willenhall, UK) at room temperature, and results were calculated from the standard curve, which was determined using Prism 5.03 software (Graphpad, La Jolla, USA).

**Figure 2.3 – Principles of ‘sandwich’ ELISA**
(a) A plate is coated with capture antibody overnight. (b) Sample is added to the plate, and the capture antibody binds to its target protein, which remains attached following plate washes. (c) Detection antibody is added, which binds the target protein at another site. (d) Streptavidin-conjugated HRP enzyme is added, which binds to the detection antibody. (e) The colourless H$_2$O$_2$ and tetramethylbenzidine substrate is added, and converted to a coloured product by the HRP enzyme. Degree of colour change is proportional to the amount of target protein present, and can be detected by a plate reader.

### 2.7.2 Cytometric bead array (CBA)

Cytokine concentrations in plasma and lavage samples were determined using mouse-specific CBA kits (BD Pharmingen, Oxford, UK; Figure 2.4). CBA was used to quantify IL-1α, IL-1β, IL-6, IL-10, TNFα, KC (CXCL1), RANTES (CCL5), MCP-1 (CCL2), IFNγ, G-CSF and CD62L, following the manufacturer’s recommended protocol with an optimised 1:5 dilution of concentrations. Standard concentration solutions, along a ten point curve, were prepared by serial dilutions of pooled flex sets. Samples and standards (2µl) were added to a rounded 96 well plate with capture bead suspension (10µl) for a 1h incubation. Subsequently, detection reagent solution (10µl) was added to each well for a further hour. The plate was spun at 200g for 5 min with a plate centrifuge (Eppendorf, Stevenage, UK) to pellet beads, the supernatant was discarded, and beads resuspended in 140µl wash buffer. Acquisition was undertaken using a BD FACSAarray$^\text{TM}$ Bioanalyzer System (BD Biosciences, Oxford, UK), and results determined using FCAP Array$^\text{TM}$ software (Soft Flow, Burnsville, Minnesota, USA).
Figure 2.4 – Principles of CBA
A CBA is used to measure the levels of multiple cytokines in a sample. Each target cytokine has a specific fluorescent bead-conjugated antibody, and PE-conjugated detection antibody. These are combined with a sample in a plate, and samples are read by a specialised flow cytometer. (a) Saturated bead for cytokine A, with high degree of PE fluorescence. (b) Bead with less bound cytokine B, with low degree of PE fluorescence. The bead’s specific red and infra-red fluorescence denote which cytokine is bound, and PE fluorescence denotes cytokine quantity.

2.8 Statistical Analysis
Data were analysed using GraphPad Prism 5.03 software and expressed as mean (±sd). Differences between groups were analysed using one-way ANOVA with post-hoc Bonferroni correction. Differences were considered statistically significant at p<0.05. Correlation between the two methods used to calculate cell numbers was analysed using the Pearson product-moment correlation coefficient.
Chapter 3

Contribution of platelets and IL-1 to neutrophil migration in LPS-induced murine peritonitis
3.1 Introduction and objectives

As discussed previously, in vitro data have shown that platelet-derived IL-1α can drive pro-inflammatory responses in mouse brain endothelial cells (MBECs), and neutrophil migration across MBEC monolayers. This thesis aims to determine if this mechanism applies in vivo. In this chapter, we aim to establish murine peritonitis as an in vivo model in which to investigate the contribution of platelets and IL-1 to the process of neutrophil migration.

Models of murine peritonitis have previously been used to investigate mechanisms of neutrophil migration (Wegmann et al. 2006; Chen et al. 2007; Kono et al. 2010). To induce peritonitis, LPS may be administered in solution directly to the peritoneal cavity. After a time, the cavity can be easily lavaged to collect any exudative cells that may be present. While this peritoneal model does not perfectly replicate the in vivo conditions of the cerebrovasculature, it does confer some advantages as a model of vascular inflammation. The peritoneum is a readily accessible anatomical space to and from which reagents can be administered and collected. This is in contrast to the skull, which requires surgical craniotomy to penetrate. The relative speed of the procedure is another advantage: much in vivo data can be collected quickly. Recent data have implicated IL-1 as a key step in sterile inflammation through use of a peritoneal inflammation model (Chen et al. 2007; Kono et al. 2010). In these reports, systemic anti-IL-1 interventions, such as monoclonal antibodies, have been shown to exert an effect on neutrophil migration into the peritoneal cavity.

The specific aims of the studies in this chapter were to determine:

1. the temporal profile of neutrophil migration in response to LPS-induced inflammation in the peritoneal cavity;
2. if platelets are required to mediate peritoneal neutrophil migration;
3. if IL-1 is required to mediate peritoneal neutrophil migration.
3.2 Methods and materials

3.2.1 Preliminary experiment
To determine the effect size of LPS in stimulating neutrophil migration in order to inform power analyses, we injected two groups of C57BL/6J mice intraperitoneally (i.p.) with vehicle (n=2) or LPS (1mg/kg, n=4) to induce peritonitis (see 2.2.1). Peritoneal lavage was performed at 4h post-injection, and lavage fluid underwent neutrophil quantification via haemocytometry, Coulter Counter and flow cytometry.

3.2.2 Characterisation of peritonitis model and platelet depletion
To characterise the temporal profile of neutrophil migration in the LPS-induced peritonitis model, C57BL/6J mice (n=30) were randomised to an intervention (LPS or vehicle) and culled at time points (4, 6 or 24h; six groups of five animals, based on power analyses). Groups were blinded to investigators during treatment and analysis. Peritoneal lavage was performed at time points of 4, 6 and 24h post-injection, and neutrophils in lavage fluid were quantified using Coulter Counter and haemocytometry measurements, combined with flow cytometry.

Anti-CD41 antibody has been used previously to deplete circulating platelets (van der Heyde et al. 2005). To determine an optimal time point for future experiments, C57BL/6J mice were injected with anti-CD41 antibody (n=5) or IgG isotype control (IgG1, κ; n=5). Tail blood samples taken at 0, 18, 24 and 48h post-injection were analysed via flow cytometry to quantify circulating platelets. To determine the effect of the antibody on circulating leukocyte populations, cardiac blood sampled prior to sacrifice at 48h post-injection underwent flow cytometric analysis to quantify the populations of various leukocytes.

3.2.3 Contribution of platelets and IL-1 to neutrophil migration in LPS-induced peritoneal inflammation
In vitro data have implicated platelets as key drivers of neutrophil transmigration (Thornton et al. 2010). To determine whether neutrophil migration is platelet-dependent in vivo, mice were randomised and treated with anti-CD41 antibody or IgG control (n=5 per group), and injected after 24h with LPS or vehicle (as 3.2.1). Circulating platelet
levels at 0, 24 and 30h were measured via Coulter Counter analysis of tail vein blood sample.

*In vitro* evidence has implicated platelet-derived IL-1α as a key driver of neutrophil transmigration (Thornton et al. 2010). To determine whether neutrophil migration is IL-1-dependent *in vivo*, wild-type (C57BL/6J) and IL-1α/β−/− double knockout mice (n=5 per group) were randomised and injected intraperitoneally with LPS or vehicle.

Treatment group allocation was blinded to investigators throughout treatment and analysis. Peritoneal lavage and cardiac blood sampling were performed 6h post-injection. Neutrophils in lavage fluid, and leukocytes and neutrophils in cardiac blood were quantified via Coulter Counter measurements and flow cytometry. Cytokine levels in cardiac blood were quantified via multiplex analysis using cytometric bead array (anti-CD41 study) and by ELISA (IL-1α/β−/− study). The detection limits of the assays were 4pg/ml (IL-1α, IL-1β, IL-6), 8pg/ml (KC) and 16pg/ml (RANTES).
3.3 Results

3.3.1 LPS stimulated neutrophil migration into the peritoneal cavity

LPS induced a significant increase in the number of neutrophils in the peritoneal cavity 4h after stimulation (p<0.05, Figure 3.1). Power analyses based on this pilot experiment indicated a group size of five for future experiments involving LPS-induced peritonitis for comparisons between vehicle- and LPS-injected mice (Appendix 7.3).

![Figure 3.1 - LPS stimulates a robust neutrophil response.](image)

Mice were dosed i.p. with vehicle (n=2) or LPS (n=4). 4h post-treatment, mice underwent peritoneal lavage. Neutrophils in lavage fluid were quantified via combination of haemocytometry and flow cytometry for Ly6G+ cells. Data are mean ±sd, *p<0.05 (t-test).

3.3.2 LPS induced significant neutrophil migration at 4, 6 and 24h

LPS induced significant migration of neutrophils into the peritoneal cavity at 4, 6 and 24h (p<0.05, Figure 3.2a). Greater variation in number of neutrophils in response to LPS was observed with increased time post-injection. Flow cytometry demonstrated an increase in the number of Ly-6G+ events (neutrophils) with the administration of LPS at all time points (Figure 3.2b,c). Furthermore, the proportion of Ly-6G+ events to all CD45+ events is increased at all time points. Thus, neutrophil migration may be investigated using LPS-induced peritonitis at time points of 4, 6 or 24h. Importantly, we have demonstrated here the validity of the *in vivo* model.
Figure 3.2 – Temporal characterisation of LPS peritonitis model.
Peritonitis was induced via intraperitoneal injection of LPS or vehicle. Peritoneal lavage was performed at 4, 6 and 24h post-injection. (a) Neutrophil counts in lavage fluid were calculated via Coulter Counter and flow cytometry. Data are mean ±sd. *p<0.05, **p<0.01 versus vehicle via one-way ANOVA with post-hoc Bonferroni correction. (b-c) Immunostaining for flow cytometry was performed on lavage fluid in (b) control animals and (c) LPS injected animals, using PE-conjugated anti-Ly-6G (neutrophil) antibody. Positively stained events indicated by an ellipse. Flow plots are representative examples from each treatment group.
3.3.3 Anti-CD41 antibody depleted circulating platelets at 18, 24 and 48h

To determine whether platelets could be depleted for a sustained period of time, we treated C57BL/6/J mice with anti-CD41 antibody or IgG isotype control. Animals treated with IgG maintained their pre-treatment levels of circulating platelets throughout the experimental period, though a non-significant decrease was seen in IgG injected animals at 48h. Animals treated with anti-CD41 antibody demonstrated a significant depletion of circulating platelets at 18 (p<0.001), 24 (p<0.01) and 48h (p<0.05) (Figure 3.3a). Injection of anti-CD41 antibody had no significant effect on the total population of leukocytes, or sub-populations of granulocytes (Gr-1+/SS), B-lymphocytes (B220+), T-lymphocytes (CD3+) and monocytes (Gr-1+/SS) (Figure 3.3b-f). Therefore, circulating platelets may be depleted via injection of anti-CD41 antibody, with no significant effect on circulating leukocytes.
Figure 3.3 – Characterisation of platelet depletion via anti-CD41 antibody.

Anti-CD41 antibody or IgG control were administered intraperitoneally. Blood was sampled via (a) tail vein sampling at 0, 18, 24 and 48h post-injection and (b-f) cardiac puncture at 48h post-injection. Quantification of (a) platelets and (b-f) leukocyte populations was carried out via flow cytometry using BD TruCOUNT™ tubes. Data are mean ±sd, all groups n=5. (a) *p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction. (b-f) non-significant via unpaired t-test.
3.3.4 Neutrophil migration occurs via a platelet-dependent mechanism in LPS-induced peritonitis

Following characterisation of anti-CD41 antibody as a tool to deplete platelets, we set out to determine whether platelets meditated neutrophil migration in LPS-induced peritonitis. Here, we depleted platelets with anti-CD41 antibody 24h before inducing peritonitis with LPS. As in 3.3.3, anti-CD41 antibody was associated with >80% reduction in circulating platelet numbers for the duration of the experiment in treated animals (p<0.001, Figure 3.4a). The reduction was also significant when compared to control animals at matched time points (p<0.01). Both IgG treated control groups revealed a small reduction in platelet numbers at 30h, which reached significance (p<0.05) in the IgG/vehicle treated group.

Flow cytometric analysis of lavage fluid replicated previous data regarding the effect of LPS, and showed a significant increase in neutrophil migration in LPS-induced peritonitis compared to controls (p<0.05, Figure 3.4b). Platelet depletion via anti-CD41 antibody almost completely abolished the recruitment of neutrophils to the peritoneal cavity in response to LPS stimulation (p<0.05, Figure 3.4b). These data show that neutrophil migration is platelet-dependent in LPS-induced peritonitis in vivo.

LPS was associated with an increase in circulating neutrophils (p<0.001, Figure 3.4b) and a decrease in circulating total leukocytes (p<0.05, Figure 3.4c) in control and thrombocytopaenic animals. Additionally, platelet depletion had no effect on circulating total or differential leukocyte (including neutrophil) counts compared to controls (Figure 3.4c).
Figure 3.4 – Neutrophil migration occurs via platelet-dependent mechanism.
Anti-CD41 antibody of IgG control was administered i.p. 24h before injection of LPS or vehicle. (a) Platelets in tail vein blood samples at 0, 24 and 30h were quantified via flow cytometry. (b) Peritoneal lavage was performed at 6h post-injection. Neutrophil quantification was performed on lavage fluid via Coulter Counter measurements and flow cytometry for Ly-6G-positive events. (c) Cardiac blood was sampled at 6h post-injection. Neutrophil and leukocyte counts were measured via flow cytometry. Data are mean±sd, all groups n=5. *p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction.
Cytokines in lavage fluid and plasma at 30h were measured via cytometric bead array. There were small, non-significant increases in the lavage levels of IL-1\(\alpha\), IL-1\(\beta\), IL-6 and CXCL1, and a significant rise in the level of the chemokine CCL5 (p<0.001, Figure 3.5a) in both control and LPS-injected animals. LPS induced a significant rise in the level of IL-1\(\alpha\) in plasma in IgG treated mice (p<0.05, Figure 3.5b). Platelet depletion via anti-CD41 antibody completely abolished this LPS-induced increase in plasma IL-1\(\alpha\) (p<0.05). Platelet depletion did not affect concentrations of other LPS-regulated plasma mediators, IL-6, CXCL1, CCL5 (Figure 3.4b), which demonstrated significant increases in LPS injected animals compared to vehicle injected controls.
Figure 3.5 – Cytokine profiles demonstrate platelet depletion selectively abrogates LPS-induced plasma IL-1α increase.

Anti-CD41 antibody of IgG control was administered i.p. 24h before injection of LPS or vehicle. Peritoneal lavage was performed at 6h post-injection. Cytokines in (a) lavage fluid and (b) plasma at 30h were quantified via cytometric bead array. Data are mean±sd, all groups n=5. *p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction.
3.3.5 Neutrophil transmigration occurs via a mechanism independent of IL-1 in LPS-induced peritonitis

To determine whether neutrophil migration in LPS-induced peritonitis was IL-1 dependent, we injected wild-type and IL-1α/β−/− double knockout mice with LPS or vehicle. Control mice replicated previous data regarding increased neutrophil migration in LPS-induced peritonitis (p<0.05, Figure 3.6a). LPS also induced significant migration of neutrophils into the peritoneal cavity in IL-1α/β−/− mice (p<0.05, Figure 3.6a). There was no difference between wild-type and IL-1α/β−/− animals. These data refute the hypothesis that peritoneal neutrophil migration is IL-1-dependent in this in vivo model. LPS induced significant thrombocytopaenia in both strains of mice (p<0.001, Figure 3.6b). Vehicle injected knockout mice had significantly more circulating platelets than their WT controls (p<0.001, Figure 3.6b).

Multiple ELISAs demonstrated the absence of an IL-1α and β response in knockout animals compared to wild-types in lavage fluid (p<0.001, Figure 3.6c), confirming their inability to secrete IL-1α and IL-1β due to genetic knockout status; although the concentration of both IL-1α and β was low in WT animals, even with LPS stimulation. Significant increases in IL-6 and CXCL1 were observed in response to LPS in both WT and knockout mice (p<0.05, Figure 3.6c). No significant effects were observed in CCL5 as a result of either intervention. The levels of IL-6, CXCL1 and CCL5 increased in response to LPS in both WT and knockout mice (Figure 3.6d).
Figure 3.6 – Neutrophil transmigration occurs via a mechanism independent of IL-1.
Wild-type or IL-1α/β−/− double knockout mice were injected i.p. with LPS or vehicle. Peritoneal lavage was performed at 6h post-injection. (a) Neutrophils present in lavage fluid were quantified via flow cytometry. (b) Circulating platelets were quantified via Coulter Counter from cardiac blood samples. Cytokines levels in (c) lavage fluid and (d) plasma were quantified via ELISA. Data are mean ±sd, all groups n=5. *p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction.
3.4 Discussion

In this chapter, we characterised an LPS-induced peritonitis model as an in vivo tool to study neutrophil migration. Using this model, we demonstrated that neutrophil migration into the inflamed peritoneum was dependent on platelets in circulation, and was independent of IL-1.

3.4.1 The response to LPS in murine peritonitis

LPS induced significant neutrophil migration at 4, 6 and 24h. We decided to carry forward the 6h time point for future experiments. This was due to the slightly larger effect size at the 6h time point, compared with 4h; and the tighter distribution and shorter experiment time compared to the 24h time point. Other studies, using a similar dose to LPS as the present study, have reported neutrophil migration of the same magnitude as our data (Renckens et al. 2006; Kornerup et al. 2010). One study investigated the dose response of neutrophil migration to LPS, and demonstrated a bell-shaped dose response curve, with a peak in neutrophil numbers in lavage fluid at a dose of 2µg/kg LPS (Werner et al. 2003), three orders of magnitude less than the dose used in the present study. Neutrophil apoptosis, induced by high levels of TNFα and IL-1β in the exudate, has been demonstrated to be responsible for reduced numbers at higher doses of LPS (Miyazaki et al. 2004); at higher doses of LPS, more neutrophils migrate, but more subsequently undergo apoptosis due to higher cytokine concentrations. Numerous other inflammatory stimuli have been used to provoke neutrophil migration into the peritoneal cavity. A notable alternative used in many studies is thioglycollate, which recruits approximately ten times more neutrophils to the peritoneal cavity compared to a similar dose of LPS, though with more variation in response (Bosse & Vestweber 1994; Borges et al. 1997; Wegmann et al. 2006).

3.4.2 Characterisation of platelet depletion via anti-CD41 antibody

Following characterisation of the neutrophil migratory response to LPS, we characterised platelet depletion via anti-CD41 antibody. Data demonstrated the efficacy of an anti-CD41 antibody in depleting platelets. Importantly depletion is sustained for 48h beyond the time of injection, meaning further interventions can be made without
the need for additional platelet depletion. Long-term depletion has been demonstrated before with this antibody, in malaria studies lasting seven days (van der Heyde et al. 2005).

There was an overall trend towards decreasing platelets in mice given IgG, although this did not reach significance at 48h. This may be due to repeated tail vein sampling; by 48h, each mouse had undergone three separate samples. While this is unlikely to be due to excessive bleeding (Hem et al. 1998), as samples were small, it may be due to platelet activation following incision of the tail vein. In future experiments, the levels of circulating platelets in control mice should be monitored for a similar trend. Figure 3.3d shows a trend towards a decrease in circulating monocytes induced by the antibody, and, although not significant, merits consideration as monocytes respond early in inflammation, can produce similar inflammatory mediators to platelets including IL-1, and may contribute to neutrophil migration. For another set of experiments outwith this thesis, we repeated the characterisation in a smaller number of mice (Appendix 7.4). This second dataset demonstrated a non-significant trend towards an increase in monocytes following anti-CD41 antibody.

CD41 is expressed on platelets, and antibodies to this ligand have previously been used to induce sustained thrombocytopenia (van der Heyde et al. 2005). Anti-CD41 antibody binds to the surface of platelets, and depletion occurs via FcR-mediated opsonisation; though it is not clear what cell types are responsible for platelet destruction, a limited role has been observed for hepatic Kupffer cells and splenic marginal macrophages (Clynes & Ravetch 1995; Nieswandt et al. 1999). Here, we used the antibody as a specific platelet intervention. CD41 is integrin alpha-IIb, and may therefore affect cell populations other than platelets. A recent paper evaluated the specificity of CD41 to the platelet lineage; it is now clear that CD41 is expressed by undifferentiated haematopoetic stem cells, as well as megakaryocytes and other platelet precursors (Robin et al. 2011). It is therefore impossible to rule out confounding effects on other bone marrow derived cells by the antibody. We took some steps to analyse this by investigating the differential count of circulating white blood cells. While these analyses did not demonstrate any significant differences, there was a trend towards
fewer circulating monocytes. It may, therefore, be prudent to monitor leukocyte levels when the antibody is used in future experiments, and to use a different anti-platelet intervention in later studies to confirm the specificity of the approach.

3.4.3 Platelets as mediators of neutrophil migration in murine peritonitis

Having demonstrated the efficacy of anti-CD41 antibody in depleting platelets, we used it as an experimental strategy to determine whether circulating platelets mediate neutrophil migration in peritonitis. Our data show that neutrophil migration in LPS-induced peritonitis required the presence of platelets in circulation. This is consistent with the findings of a study of different aspects of platelet function in zymosan-induced peritonitis (Kornerup et al. 2010). Zymosan is a PAMP derived from the yeast cell wall, as opposed to LPS, which is found in the cell wall of Gram-negative bacteria. Neutrophil migration into the inflamed peritoneum following zymosan was abrogated by the depletion of platelets with busulfan, a chemotherapeutic agent. Further, neutrophil migration was reinstated by the intravenous infusion of platelet-rich plasma, but not platelet-poor plasma, into mice treated with busulfan. The authors proceeded to test the effects of antibodies to selectin molecules, in an attempt to discover the mechanism by which platelets supported neutrophil migration. Anti-P-selectin antibody, which acts against the major selectin class expressed by platelets had no effect; yet, anti-PSGL1 antibody blocked neutrophil migration into the peritoneum. Blocking L-selectin had no effect, while E-selectin blockade also reduced migration. The role for PSGL1 as a key molecular mediator of neutrophil migration into the peritoneum had been observed before in response to thioglycollate (Borges et al. 1997; Yang et al. 2002; Hicks et al. 2003). A role for PSGL1 does not necessarily implicate platelets in neutrophil migration, as platelets express P-selectin, rather than its ligand PSGL1. Additionally, PSGL1 is a ligand for all P-, L- and E-selectin, so PSGL1 blockade is not specific to one class of cell. However, there may be a supportive role for platelets in promoting the expression of PSGL1 on migrating neutrophils. Activated neutrophils form a ‘cap’ of PSGL1 on one face of the cell surface, a topographical redistribution of PSGL1 that is facilitated by platelets in co-culture (Itoh et al. 2007). The question remains, therefore, as to precisely how platelets mediate neutrophil migration; and it
also remains a possibility that a soluble mediator released by platelets, such as IL-1α, could facilitate PSGL1 expression (Todoroki et al. 1991).

LPS exerted the same effect in both control and platelet-depleted mice in terms of circulating leukocytes. Total leukocyte numbers fell significantly, potentially a result of increased adherence to the vascular endothelium and/or redistribution of lymphocytes, the major leukocyte subclass in murine blood, to lymph nodes. Total neutrophils rose in both groups, possibly reflecting increased mobilisation from the bone marrow and/or release of the marginating pool. This pattern has been observed before, with the increase in the number of circulating neutrophils being dependent on the dose of LPS (Werner et al. 2003). A time course investigation of this pattern demonstrated that 15 min after LPS injection, there is a significant fall in neutrophil numbers, and at 30 min, a doubling of baseline neutrophils (Miyazaki et al. 2004), a pattern consistent with the absolute numbers of neutrophils in our data.

The cytokine response we observed, with larger rises in IL-6 and chemokines has been observed before (Renckens et al. 2006; Privratsky et al. 2012). One paper demonstrated that cytokine levels were proportional to the dose of LPS used (Privratsky et al. 2012). Compared to this paper, we used a small dose of LPS, which may explain why the small increases in cytokine concentration in lavage fluid did not reach significance. Alternatively, our use of 5ml of fluid to lavage the peritoneal cavity may result in underestimation of the concentration of cytokines.

Plasma cytokines in our study exhibited a similar response to LPS as previously observed, with larger increases in IL-6 and TNFα, than either IL-1 ligand (Renckens et al. 2006). Platelet depletion abrogated the increase in plasma IL-1α induced by LPS. Clinical and ex vivo experiments have shown platelets to be a potent source of IL-1α (Boilard et al. 2010; Thornton et al. 2010). This effect was not seen in pro-inflammatory cytokines downstream of IL-1α, such as IL-6, nor in chemotactic cytokines such as KC and RANTES. This may suggest that other upstream cytokines may regulate these mediators. The specificity of this approach suggests that platelets may be an important source or trigger of IL-1α in this model. Importantly, we did not observe an effect of
anti-CD41 antibody on the plasma levels of chemokines or on neutrophil numbers, as either of these factors could have directly affected neutrophil migration; CXCL1 blockade has been shown to significantly reduce neutrophil recruitment after LPS-induced peritonitis (McColl & Clark-Lewis 1999).

Together, these data indicate that the effects of platelet depletion are likely acting at the local site of leukocyte vascular extravasation rather than on mobilisation of neutrophils into the circulation (in terms of circulating numbers and chemotactic stimulus), and, given the plasma cytokine data, may point to platelet-derived IL-1α as a candidate molecular mediator of localised vascular inflammation in vivo.

3.4.4 IL-1 as a mediator of neutrophil migration in murine peritonitis

To evaluate the role of IL-1 in neutrophil migration in LPS-induced peritonitis, we used IL-1α/β−/− double knockout mice, constitutively lacking both IL-1 ligands. There was no difference between the wild-type and IL-1α/β−/− double knockout mice in their response to LPS. This refutes the hypothesis that platelet-derived IL-1α is an important driver of vascular inflammation in a peritoneal model of vascular inflammation. Control groups showed similar responses as observed previously, demonstrating both significant neutrophil migration and cytokine responses to LPS. This was particularly marked in relation to IL-6 and the chemokines CXCL1 and CCL5. There was no significant difference in levels of chemokines across mouse strains. LPS induced significant thrombocytopenia in both strains of mice, which is widely reported in the literature, and was observed at 30h in IgG injected mice in the platelet depletion study (Davis et al. 1960; Vincent et al. 2002).

There are no published studies investigating the role of IL-1 in mediating neutrophil migration in LPS-induced peritonitis with which to compare our finding. However, studies involving sterile inflammatory stimuli in the peritoneum have demonstrated a clear role for IL-1 in promoting neutrophil migration (Chen et al. 2007; Kono et al. 2010). This contrasts with our results in the LPS model, suggesting a difference in the requirement of IL-1 between inflammatory stimuli; mechanisms in relation to neutrophil migration.
3.4.5 Neutrophil migration in LPS-induced peritonitis

Neutrophil migration in LPS-induced peritonitis was dependent on platelets and independent of IL-1. The overall aim of this thesis is to determine whether platelet-derived IL-1α plays a role in vascular inflammation, based on the findings of Thornton et al. (2010) in the cerebrovasculature. While IL-1 drove neutrophil migration across cultured MBECs, IL-1 was not necessary for neutrophils to migrate into the peritoneum following LPS stimulation. There are key differences between the in vivo model used here and the in vitro mouse brain endothelial cell experiments in Thornton et al.’s experiments that prompted our experiments. Two notable differences are the nature of the inflammatory stimulus, and the site of inflammation.

Studies have established IL-1 as necessary mediator of neutrophil migration in sterile peritonitis (Chen et al. 2007; Kono et al. 2010). Cerebral inflammation is more commonly sterile than in the peritoneum, and IL-1 is implicated in neutrophil migration in the brain (McColl et al. 2007). IL-1 may therefore play an important role in vivo in mediating neutrophil migration in sterile inflammation consistently across different vascular beds. Our use of LPS, a PAMP, may therefore explain the difference in requirement for IL-1. An alternative explanation for the differential requirement for IL-1 is a difference in endothelial cells: MBECs may respond differently to inflammatory mediators to the endothelial cells of other vascular beds. Therefore, it is important to determine whether IL-1, and indeed platelets, are required to mediate neutrophil migration in other vascular beds, including the brain.

Thornton et al. (2010) demonstrated that it was IL-1α specifically derived from platelets that was an important driver of neutrophil migration. Anti-CD41 antibody treatment revealed that neutrophil migration in the peritonitis model was dependent on platelets. Platelet depletion also caused abrogation of the plasma increase in IL-1α induced by LPS. This means that platelets may be an important source or stimulus of the plasma IL-1α response in this model, but given the data from IL-1 knockout mice, this response does not seem critical to neutrophil migration.
3.9 Conclusion

The aims of the studies in this chapter were to determine:

1. the temporal profile of neutrophil migration in response to LPS-induced inflammation in the peritoneal cavity;
2. if platelets are required to mediate peritoneal neutrophil migration;
3. if IL-1 is required to mediate peritoneal neutrophil migration.

LPS was injected i.p. to stimulate neutrophil migration. This formed a reproducible model, in which the efficacy of various interventions could be tested. The migration of neutrophils was dependent on platelets, yet independent of IL-1.

Thornton et al. (2010) defined a role for platelet-derived IL-1α as a key driver of cerebrovascular inflammation and neutrophil migration. Mechanisms of neutrophil migration appear context dependent, and further studies aim to determine whether IL-1 is required for neutrophil migration in other vascular beds.
Chapter 4

Contribution of platelets and IL-1 to neutrophil migration in LPS-induced air pouch inflammation and acute lung injury
4.1 Introduction

In Chapter 3 we investigated the role of platelets and IL-1 in neutrophil migration in a peritonitis model. It had previously been demonstrated that platelet-derived IL-1α was a key driver of the inflammation of cultured mouse brain endothelial cells (MBECs), and neutrophil migration across MBEC monolayers (Thornton et al. 2010). In Chapter 3, the migration of neutrophils into the peritoneal cavity following LPS injection was dependent on platelets, yet independent of IL-1. This in vivo evidence contrasts with the in vitro work that demonstrated a clear role for IL-1 in the inflammation of cerebrovascular endothelium. While the difference may generally relate to the more complex immunological and haematological conditions in vivo, it also could reflect differential contributions of specific mediators to inflammatory processes in different vascular beds. In support, there is indirect evidence, across published studies, that vascular inflammatory processes may differ between tissues. In particular, one study noted that platelet IL-1 stimulated different cytokine responses from endothelial cell of different tissues in vitro (Hawrylowicz et al. 1991). Therefore, given the results from the previous chapter and published data, a key question to address is whether the contribution of platelets and IL-1 to driving acute inflammation in vivo differs depending on the vascular bed/tissue.

This chapter focusses on two further in vivo models of extra-cerebral vascular inflammation to establish if the platelet-dependent and IL-1-independent effects observed in the peritoneum (Chapter 3) are common to other extra-cerebral tissues.

The aims of the studies in this chapter were to determine:

1. if platelets are required to mediate neutrophil migration into a subcutaneous air pouch;
2. if IL-1 is required to mediate neutrophil migration into a subcutaneous air pouch;
3. if platelets are required to mediate neutrophil migration into the broncho-alveolar space;
4. if IL-1 is required to mediate neutrophil migration into the broncho-alveolar space.
4.2 Methods and materials

4.2.1 Air pouch inflammation model

The first reported use of a murine dorsal air pouch was in 1953, when Selye described how a cavity could be created, via the insufflation of air, to study inflammatory processes (Selye 1953; Colville-Nash & Lawrence 2003). In the version of the air pouch model used here, we injected sterile air over seven days (see 2.2.2), as described previously (Kukulski et al. 2007). This has been demonstrated to produce an artificial cavity lined with granulation tissue (Edwards et al. 1981). The subsequent injection of inflammatory stimuli into the air pouch allows the study of inflammatory reactions via aspiration of the resulting exudate and quantification of migrated cells, including neutrophils (Sin et al. 1986; J Dawson et al. 1991; Colville-Nash & Lawrence 2003).

The model has been widely used in studying inflammatory processes ranging from determining physiological mechanisms to profiling the effects of new medications for crystal arthropathy (Sedgwick et al. 1985; Cronstein 1995; Ferrándiz et al. 1996; Nickerson-Nutter & Medvedeff 1996; Perretti et al. 1996). A distinguishing feature of the air pouch model is the localisation of the inflammatory stimulus; LPS almost exclusively being confined to the air pouch (Miller et al. 1997b; Cartmell et al. 2001).

Air pouches were created as per 2.2.2. To determine the effect size for power analyses of LPS in stimulating neutrophil migration into the air pouch, we injected two groups of C57BL/6J mice with vehicle (n=5) or LPS (1mg/kg, n=5) to induce inflammation.

For all experiments, air pouch lavage was performed at 6h post-LPS challenge, and neutrophil accumulation in lavage fluid quantified via Coulter Counter and flow cytometry. Neutrophils and leukocytes in cardiac blood were quantified by flow cytometry. Cytokine levels in lavage fluid and cardiac blood were quantified by multiplex analysis using CBA.

To determine whether neutrophil migration is platelet-dependent in the *in vivo* air pouch model, mice were treated with anti-CD41 antibody or IgG control as described previously, and the air pouch injected after 24h with LPS or vehicle. Four groups of five C57BL/6J mice were used; groups were randomised and blinded to investigators.
throughout treatment and analysis. Circulating platelet levels at 30h were measured via Coulter Counter and flow cytometric analysis of cardiac blood samples.

![Diagram showing anti-CD41 Ab IgG control and LPS Vehicle groups with 24h and 6h cull time points]

To determine whether neutrophil migration is IL-1-dependent in the air pouch model, wild-type C57BL/6J and IL-1α/β−/− double knockout mice were injected via a dorsal air pouch with LPS (or vehicle). Four groups of five animals were used. Groups were randomised and blinded to investigators during the procedure and analysis.

4.2.2 Broncho-alveolar inflammation model

Neutrophils are early mediators of LPS-induced lung inflammation (Williams et al. 1993). The second model we use in this chapter involves exposure to aerosolised LPS (see 2.2.3). LPS inhalation has previously been used to investigate neutrophil migration into broncho-alveolar lavage fluid (BALF) and lung tissue (Skerrett et al. 2004; Reutershan et al. 2005). Designed as a model of acute lung injury (ALI), and specifically of infection with gram-negative bacteria, the protocol can also be used to study more fundamental inflammatory processes. The response to aerosolised LPS includes increased expression of adhesion molecules by endothelial cells of and release of cytokines, characteristics of the in vitro model under investigation (Lorenz et al. 2001). LPS inhalation also increases airway resistance (Lorenz et al. 2001; Reutershan et al. 2005).

Broncho-alveolar inflammation was induced as per 2.2.3. To determine whether neutrophil migration is platelet dependent, mice were treated with anti-CD41 antibody or IgG control as described previously, and 24h later were exposed to nebulised LPS (3mg/ml stock) or vehicle. Four groups of five C57BL/6J mice were used. To determine whether neutrophil migration is IL-1 dependent, IL-1α/β−/− double knockout mice and wild-type C57BL/6J mice were exposed to nebulised LPS or vehicle. Again, four groups of five mice were used. These two experiments were performed simultaneously.
While the order of treatments was randomised, exposure to LPS and vehicle was performed in two batches of 20. All groups were blinded to investigators throughout treatment and analysis. Broncho-alveolar lavage fluid (BALF) and cardiac blood sampling were performed 6h post-injection. Neutrophils in BALF, and leukocytes and neutrophils in cardiac blood were quantified via Coulter Counter measurements and flow cytometry. Circulating platelet levels at 30h were measured via Coulter Counter and flow cytometric analysis of a cardiac blood sample. Cytokine levels in BALF and cardiac blood were quantified via multiplex analysis using CBA.

4.3 Results

4.3.1 LPS stimulates neutrophil migration in an air pouch model

LPS injection into a dorsal air pouch stimulated a significant four-fold increase in the number of migrated neutrophils present in pouch lavage fluid at 6h when compared to vehicle injection (p<0.01, Figure 4.1a). LPS induced a small rise in the number of circulating neutrophils, though this did not reach significance, and a significant fall in the number of circulating leukocytes (p<0.01, Figure 4.1b). Power analyses based on this pilot experiment indicated a group size of five for future studies involving comparisons between vehicle- and LPS-injected mice, representing a similar effect size as also used in the peritonitis model. Calculation of power analyses is presented in Appendix 7.3.
4.3.2 Neutrophil migration occurs via a platelet-dependent mechanism in LPS-induced air pouch inflammation

Flow cytometric analysis of lavage fluid again revealed neutrophil transmigration in LPS-induced air pouch inflammation in IgG-treated animals (p<0.05, Figure 4.2a). Platelet depletion via anti-CD41 antibody abrogated the recruitment of neutrophils to the peritoneal cavity in response to LPS stimulation (p<0.05, Figure 3.4a). These data support the hypothesis that neutrophil migration is platelet-dependent in this model. Anti-CD41 antibody was associated with a >80% reduction in circulating platelet numbers at 30h in treated animals compared to IgG controls (p<0.001, Figure 4.2b), as previously demonstrated in Chapter 3. LPS was associated with no change in circulating neutrophils (Figure 4.2c) and a decrease in circulating leukocytes in control (p<0.05) and thrombocytopenic animals (ns).

Figure 4.1 - LPS stimulates a neutrophil invasion to a subcutaneous air pouch
Mice were injected directly into a dorsal air pouch with vehicle or LPS. 6h post-treatment, mice underwent peritoneal lavage. (a) Neutrophils in air pouch lavage fluid were quantified via combination of Coulter counter and flow cytometry for Ly6G+ cells. (b) Neutrophils and total leukocytes in cardiac blood were quantified via combination of Coulter counter and flow cytometry for Ly6G+ and CD45+ cells. Data are mean ±sd, all groups n=3, **p<0.01 via t-test.
Multiplex analysis of cytokines and chemokines in lavage fluid 6h after LPS challenge revealed a similar pattern to that observed previously in the peritonitis model. Significant increases were observed in the concentrations of IL-1β, TNFα, IL-6, CXCL1 and CCL5 after LPS injection, and there was no difference between platelet depleted mice and controls (Figure 4.3). A trend toward an increase was observed for IL-1α levels in both thrombocytopenic and control animals, but this did not reach statistical significance.
Figure 4.3 – LPS induces a widespread cytokine response in air pouch lavage fluid.
Anti-CD41 antibody or IgG control was administered i.p. 24h before injection of LPS or vehicle into a dorsal air pouch. Cytokines levels in air pouch lavage fluid were quantified via CBA. Data are mean ±sd, all groups n=5. *p<0.05, **p<0.01 via one-way ANOVA with post-hoc Bonferroni correction.
Analysis of cytokines and chemokines in plasma revealed significant increases in the levels of TNFα, IL-6, KC and RANTES after LPS injection, with no difference between anti-CD41 treated animals and controls (Figure 4.4). There was no change in the levels of IL-1β associated with LPS injection. LPS provoked a rise in plasma IL-1α concentration in control mice, and platelet depletion completely abolished the LPS-induced increase in plasma IL-1α (p<0.05, Figure 4.4).
Figure 4.4 – Selective abrogation of the plasma IL-1α response to LPS following platelet depletion. Anti-CD41 antibody of IgG control was administered i.p. 24h before injection of LPS or vehicle into a dorsal air pouch. Cytokines levels in plasma were quantified via CBA. Data are mean ±sd, all groups n=5. *p<0.05, **p<0.01 via one-way ANOVA with post-hoc Bonferroni correction.
4.3.3 Neutrophil transmigration occurs via mechanism independent of IL-1 in a subcutaneous air pouch

WT mice replicated previous data regarding increased neutrophil transmigration in the LPS-induced air pouch inflammation model (p<0.05, Figure 4.5a). No difference was observed in LPS-induced accumulation of neutrophils into the air pouch; and there was no difference between wild-type and IL-1α/β⁻/⁻ animals’ neutrophil response to LPS. These data suggest that neutrophil migration is IL-1-independent in this in vivo model, however there was no statistical increase after LPS in IL-1α/β⁻/⁻ mice. LPS was associated with a small increase in circulating neutrophils in WT mice (ns, Figure 4.5b), and no difference in KO animals. Both strains exhibited a decrease in circulating leukocytes in control animals, which reached significance in IL-1α/β⁻/⁻ mice (p<0.05, Figure 4.5b).
Figure 4.5 – Neutrophil migration occurs via a mechanism independent of IL-1 in an in vivo air pouch model of inflammation.

Wild-type and IL-1αβ−/− double knockout mice underwent injection of LPS or vehicle into a dorsal air pouch. (a) Lavage was performed 6h post-injection. Neutrophil in lavage fluid were quantified via Coulter Counter and flow cytometry for Ly-6G+ events. Cardiac blood was sampled at 6h post-injection. (b) Neutrophil and leukocyte counts were measured via flow cytometry. Data are mean±sd, all groups n=5. *p<0.05 via one-way ANOVA with post-hoc Bonferroni correction.
4.3.4 Neutrophil migration in LPS-induced ALI is independent of platelets

To determine whether neutrophil migration was dependent on platelets in ALI, we exposed control and thrombocytopenic mice to aerosolised LPS or vehicle. Following LPS inhalation, mice treated with IgG demonstrated a significantly increased number of neutrophils in BALF (p<0.001, Figure 4.6a). Mice that underwent platelet depletion via anti-CD41 antibody also exhibited a significantly increased number of neutrophils in BALF after LPS exposure (p<0.001, Figure 4.6a). There was no difference between the IgG and anti-CD41 treated groups, signifying that neutrophil migration is independent of platelets in this model of acute lung injury under the conditions used. LPS exposure did not affect circulating levels of leukocytes or neutrophils in either IgG or anti-CD41 treated mice (not shown). Treatment with anti-CD41 antibody caused a >90% depletion of platelets in both LPS and vehicle exposed animals (p<0.001, Figure 4.6b).

Measurement of cytokines in BALF demonstrated significant rises in IL-1α, IL-1β, IL-6, TNFα, CXCL1 and CCL5 (p<0.01, Figure 4.6c). In thrombocytopenic animals a similar pattern was observed, though the increase in IL-1β did not reach significance. Levels of CXCL1 and CCL5 were significantly higher in thrombocytopenic animals than controls (p<0.05, Figure 4.5c), which may indicate a larger chemotactic signal in these animals.

Quantification of cytokines in plasma 6h after LPS exposure demonstrated no effect on levels of IL-1α, IL-1β, IL-6, and TNFα, while significant increases were seen in levels of chemokines CXCL1 and CCL5 (p<0.05, Figure 4.6d). The same pattern was observed in thrombocytopenic animals, suggesting that platelet depletion has no effect on the plasma cytokine response to LPS.
Figure 4.6 - Neutrophil migration occurs independently of platelets in a model of acute lung injury. Anti-CD41 antibody or IgG control was administered i.p. 24h before exposure to LPS or vehicle. Lung airways were lavaged 6h after exposure to nebulised LPS. (a) Neutrophils in lavage fluid and (b) platelets in circulation were quantified via flow cytometry. Cytokines in (c) lavage fluid and (d) plasma were measured via cytometric bead array. Data are mean±sd, all groups n=5. *p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction.
4.3.5 Neutrophil migration in LPS-induced ALI is independent of IL-1

To determine whether neutrophil migration in ALI was dependent on IL-1, we exposed WT and IL-1α/β⁻/⁻ knockout mice to aerosolised LPS or vehicle. Wild-type mice exposed to LPS had significantly more neutrophils in BALF compared to vehicle controls (p<0.05, Figure 4.6a). IL-1α/β⁻/⁻ double knockout mice also had significantly more neutrophils in BALF following LPS exposure compared to their vehicle exposed controls (p<0.01, Figure 4.6a). There was no difference between the groups of WT and IL-1α/β⁻/⁻ mice indicating that neutrophil migration in this model of acute lung injury was independent of IL-1. In both WT and knockout mice, there was a non-significant trend towards an increase in the total numbers of circulating leukocytes (Figure 4.6b) and no effect on circulating neutrophil levels (not shown).

Measurement of cytokines in the BALF of WT mice following LPS exposure demonstrated significant rises in IL-1α, IL-6, TNFα and CCL5 (p<0.001, Figure 4.6c), and non-significant rises in IL-1β and CXCL1. As expected, there was no IL-1α or IL-1β response in the BALF of double knockout mice to LPS, though there were significant rises in IL-6, TNFα, CXCL1 and CCL5 (p<0.001, Figure 4.6c). Levels of CXCL1 were significantly higher in knockout animals than WT (p<0.001), which may represent a larger degree of chemotaxis for neutrophils in IL-1α/β⁻/⁻ mice.

Quantification of cytokines in the plasma of WT and knockout mice showed no response in the levels of IL-1α, IL-1β, IL-6 and TNFα to LPS (Figure 4.6d). A significant increase was observed in CCL5 in both WT and knockouts (p<0.001, Figure 4.6d). Following LPS, the double knockout mice showed a significantly higher level of the chemokine CXCL1 in plasma compared to wild-type animals.
Figure 4.7 - Neutrophil migration occurs independently of IL-1 in a model of acute lung injury. Mice were exposed to aerosolised LPS or vehicle. (a) Neutrophils in lavage fluid and (b) leukocytes in circulation were quantified via flow cytometry. Cytokines in (c) lavage fluid and (d) plasma were measured via cytometric bead array. Data are mean±sd, all groups n=5. *p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction.
4.4 Discussion

In this chapter, we characterised an LPS-induced air pouch inflammation model as a tool to study neutrophil migration. Using this model, we demonstrated that neutrophil migration into the inflamed air pouch required the presence of platelets in circulation, and was independent of IL-1. In an *in vivo* model of lung inflammation induced by LPS inhalation, neutrophil migration into the broncho-alveolar space was independent of IL-1, as shown in the air pouch and peritoneum. In contrast, however, neutrophil migration in the lung was also platelet-independent.

4.4.1 The response to LPS in the murine air pouch

While no studies have investigated the role of IL-1 or platelets in neutrophil migration in an LPS air pouch model, the effects of LPS on neutrophil migration (Gambero *et al.* 2003; Vasileiadou *et al.* 2010), and cytokine responses (Miller *et al.* 1997a; 1997b; Cartmell *et al.* 2000; 2001) in the air pouch are well characterised, though the role of IL-1 or platelets in response has not been reported.

In our version of the air pouch model, we used an LPS dose of 1mg/kg and a time point of 6h. We demonstrated that a consistent number of neutrophils migrated into the air pouch following this stimulus: approximately 2,000 neutrophils/ml lavage fluid (Figures 4.1a, 4.2a, 4.5a). A study using a dose of 3mg/kg LPS found a mean response of approximately 7,000 neutrophils/ml, a number of the same magnitude as our findings (Vasileiadou *et al.* 2010). The same study evaluated the temporal profile of neutrophil migration into the pouch and reported a peak in pouch neutrophil numbers at 6h. This provides support for our decision to use a 6h time point, chosen so as to be compatible with the peritonitis model in Chapter 3. Another study investigated the inflammatory response to inoculation of the air pouch with *Helicobacter pylori*, a Gram negative bacterium (Gambero *et al.* 2003). The use of a Gram negative organism is not a direct correlate of LPS stimulation alone; however, the LPS present in the organism’s cell wall drives inflammatory responses via the same pattern recognition receptors, and is the key trigger of the immune response to infection by Gram negative organisms (Fenton & Golenbock 1998; Takeuchi *et al.* 1999; Medzhitov 2001). The same temporal pattern of neutrophil migration was observed following inoculation with *H. pylori*: a peak at 6h.
(Gambero et al. 2003). Other studies have evaluated the effect of other stimuli in an air pouch model using thioglycollate and zymosan injections (Bouma et al. 2005; Mamuk & Melli 2007). Similar patterns of neutrophil migration into the pouch were observed here, though a later peak at 8h was seen in response to thioglycollate injection (Bouma et al. 2005).

Other studies that used LPS as an inflammatory stimulus in the air pouch model reported significant responses in pouch IL-1α, IL-1β and IL-6 levels peaking 5-8h following injection (Cartmell et al. 2001; Vasileiadou et al. 2010). We replicated significant IL-1β and IL-6 responses, but the increase in IL-1α seen in our study did not reach significance. Absolute levels of cytokine are different between studies, which may be explained by different dosages of LPS administered. In addition to neutrophil migration, Gambero et al. (2003) measured the cytokine response to *H. pylori* infection. IL-1β and TNFα levels in the pouch peaked at 6h, comparable with our data. One paper investigated in detail differences in cytokine responses between the local air pouch environment and the circulation following LPS injection in rats (Cartmell et al. 2000). They observed large responses in IL-1β and IL-6 in the air pouch, and only a significant IL-6 rise in plasma; a pattern replicated in our data (Figures 4.3 & 4.4), with no change in the level of circulating IL-1β following LPS injection. An important observation from the literature is that LPS remains in the air pouch: following LPS injection in an air pouch model, no LPS was detectable in plasma and approximately 85% of the injected amount could be recovered by lavage at 8h; the 15% shortfall being attributed to phagocyte action (Miller et al. 1997b; Cartmell et al. 2001). This is very different to the peritonitis model, where injected LPS is likely to be close to 100% bioavailable (Banks & Robinson 2010). This difference in absorbance is likely to explain the different plasma IL-1β response between the two models.

In a similar pattern to LPS peritonitis in Chapter 3, the injection of LPS into an air pouch caused a significant reduction in the number of circulating leukocytes; this has been explained previously in terms of adhesion to the vasculature or redistribution of lymphocytes to lymph nodes (see Chapter 3 discussion). The increase in circulating neutrophils caused by LPS was not as pronounced in the air pouch model, and did not
reach significance. Levels of the chemokine CXCL1 in plasma were comparable across both models. The difference could be explained by the lack of systemic LPS absorbance seen in the air pouch model.

4.4.1.1 Platelets as mediators of neutrophil migration into the murine air pouch
Platelets were necessary for the migration of neutrophils into the air pouch, replicating the findings from Chapter 3 in peritoneal inflammation. Importantly, results showed the same numbers of circulating leukocytes and neutrophils in control and thrombocytopenic mice, and the same level of chemotaxis from CXCL1 (Figures 4.2, 4.3). No studies have investigated platelets as a mediator of neutrophil migration in this model, so our data represent the first evidence in support of platelet-dependent mechanisms in this model of local inflammation.

Interestingly, one study investigated the effect of aspirin in zymosan-induced air pouch inflammation (Mamuk & Melli 2007). Aspirin, is a non-steroidal anti-inflammatory with potent anti-platelet effects, mediated by prevention of the synthesis of platelet-derived thromboxane A2 (TXA2). TXA2 is a potent platelet-activating factor, important in driving platelet and leukocyte recruitment to sites of inflammation (Ueno et al. 2011). Aspirin had no effect on neutrophil migration (Mamuk & Melli 2007). Coupled with the clear role identified for platelets in this chapter, it may suggest that an aspect of platelet function other than that performed by TXA2 may drive neutrophil recruitment. The role of other platelet-mediated mechanisms in neutrophil migration has been investigated more extensively in lung inflammation (see 4.4.5).

Our analysis of cytokines also revealed interesting patterns. In a repeat of the observation in the peritonitis model (Chapter 3), platelet depletion abrogated the increase in plasma IL-1α induced by LPS in the air pouch. This effect was, again, not seen in levels of other pro-inflammatory cytokines, nor in the chemokines CXCL1 and CCL5. The specificity of this pattern for IL-1α suggests that platelets may either be an important source of IL-1α, or trigger its production, in this model. Given that, once injected, no LPS escapes the air pouch (Miller et al. 1997b; Cartmell et al. 2001), any cytokine response in plasma is likely to have come from cells in circulation responding
to the inflammatory process triggered by LPS in the air pouch. This means that the IL-1α response seen in plasma originates in the circulation and occurs downstream of the initial inflammation in the air pouch. While platelets are necessary for the IL-1α response in plasma, there must be another source for IL-1α in the air pouch as this was unaffected by anti-CD41 antibody. A likely candidate cellular source of IL-1α may be macrophages, which perform a similar role in the peritoneum (Kono & Rock 2008).

4.4.1.2 IL-1 as a mediator of neutrophil migration into the murine air pouch

There was no difference between the wild-type and IL-1α/β−/− double knockout mice in terms of neutrophil migration. Thus, neutrophils migrate independently of IL-1 in air pouch inflammation induced by LPS; a pattern which we have also demonstrated in LPS-induced peritonitis (Chapter 3). No other studies have investigated the role of IL-1 in mediating neutrophil migration in this model.

Neutrophil migration into the air pouch has, however, been blocked by antibodies to TNFα (Arreto et al. 1997). The authors identified alveolar macrophages as key effector cells via inhibition of neutrophil migration through selective depletion of macrophages in the pouch with lipsosome-encapsulated clodronate, and systemic depletion with cyclohexamide. Both depletion methods resulted in the abrogation of TNFα activity in the pouch. In cyclohexamide treated animals, TNFα activity and neutrophil migration were recovered following replenishment of pouches with alveolar macrophages. Our data show a large and statistically significant TNFα response to LPS in the air pouch lavage fluid, which is consistent with the findings of Arreto et al. (1997). To further explore the role of TNFα, another study injected carrageenan into air pouches (García-Ramallo et al. 2002). Carrageenan is a polysaccharide derived from seaweed and, like LPS, it triggers inflammation in mammals via activation of TLR4 (Bhattacharyya et al. 2008). Garcia-Ramallo et al. (2002) demonstrated that at 2h post-injection, neutrophils had not yet migrated into the pouch, yet there was a significant increase in pouch levels of TNFα and many chemokines. In combination with the data from another study (Tessier et al. 1997), these data implicate TNFα derived from macrophages resident in the air pouch as the cells responsible for initialising inflammation. The study could not rule out the potential involvement of IL-1 at this stage in driving neutrophil migration;
though we have now demonstrated the process is independent of both IL-1 ligands. Combining the data from Arreto et al. (1997), Garcia-Ramallo et al. (2002) and our study, there appears a key role for TNFα, and not IL-1, in driving neutrophil migration into the air pouch. A key role for IL-6 remains a possibility. IL-6 levels are raised at early time points in the air pouch (Cartmell et al. 2000; 2001), and evidence exists that it plays an important role in leukocyte recruitment (Romano et al. 1997). IL-6 levels significantly increased in response to LPS in our study, which is consistent with this hypothesis.

In Chapter 3, we discussed that there was a difference in the requirement for IL-1 between different triggers of inflammation in the murine peritonitis model: LPS-induced neutrophil migration was independent of IL-1 in our hands, while sterile inflammation has been demonstrated to be IL-1 dependent (Chen et al. 2007; Kono et al. 2010). Two studies have evaluated the role of IL-1 in sterile inflammation in an air pouch model (Yang et al. 2002; Jin et al. 2011). Particles of polyethylene stimulated leukocyte migration into the air pouch, an effect abrogated by the introduction of retroviruses encoding IL-1Ra (Yang et al. 2002). Further evidence of the involvement of IL-1 involved the use of mice deficient in components of the NLRP3 inflammasome, specifically NLRP3−/− and ASC−/−, and caspase-1 knockout mice (Jin et al. 2011). All strains of knockout mice exhibited significantly less neutrophil infiltration and lower air pouch IL-6 levels compared to controls. Therefore, in contrast to our findings in LPS-mediated inflammation, neutrophil recruitment into an air pouch during sterile inflammation appears dependent on IL-1.

4.4.2 Neutrophil migration in ALI

In the broncho-alveolar model, neutrophils have to migrate through the vascular endothelium, through the lung tissue and across the alveolar epithelium to reach the broncho-alveolar space, where they can be detected in BALF. The lung tissue through which neutrophils must migrate is highly specialised to facilitate gas exchange, and as a result there may be differences in the migration of neutrophils in this context (Doerschuk 2001). There is evidence that neutrophil migration in the lungs occurs in capillary beds, as opposed to post-capillary venules in other vascular beds (Lien et al.
1987; 1991; Downey et al. 1993), and that the fibroblasts of the lung parenchyma and the airway epithelium exert a unique effect on neutrophil movement (Walker et al. 1995; Behzad et al. 1996).

Models of acute lung injury aim to simulate the histological pattern observed clinically, namely lung oedema, endothelial and epithelial injury, and neutrophil influx into the lung parenchyma and broncho-alveolar space. This is different to the air pouch model, which does not aim to replicate a clinical syndrome. There is significant heterogeneity in inflammatory stimuli used in models of ALI, with models including inhalation of hydrochloric acid or LPS with and without zymosan, systemic injection of LPS, transfusion-related ALI and allergic ovalbumin sensitisation-provocation models. Of greater importance to this study, there is also heterogeneity in how inhalation is facilitated, with intubation of the trachea, tracheostomy and nebulisation chamber all described in the literature (Wheeldon et al. 1992; Miotla et al. 1996; Pitchford et al. 2005; Zarbock et al. 2006; Grommes et al. 2012).

LPS inhalation has repeatedly been shown to cause neutrophil recruitment into the broncho-alveolar space (Wheeldon et al. 1992; Lorenz et al. 2001; Skerrett et al. 2004; Reutershan et al. 2005; Grommes et al. 2012). There is substantial variation between studies in the number of neutrophils that migrate into BALF following LPS. This may be explained by heterogeneity in the methods used. For example, LPS inhalation via intubation of the trachea caused a neutrophil response three orders of magnitude greater than observed in this study (Wheeldon et al. 1992). Although we used a nebulised solution of 3mg/ml LPS, the concentration reaching the lungs of the mice would have been substantially lower, and a function of nebuliser activity and chamber volume. These factors are impossible to compare across studies, as they are not included in written reports.

The cytokine response to LPS in our model was marked in BALF, with significant increases in IL-1α, IL-1β, IL-6, TNFα and the chemokines CXCL1 and CCL5 (Figures 4.5, 4.6). This is comparable to data in experiments designed to characterise the lung cytokine response to LPS (Johnston et al. 1998). In contrast, the response to LPS in the
plasma was restricted to the chemokines CXCL1 and CCL5, some of which may have been produced by the pulmonary vascular endothelium (Beck et al. 1999). This is a different response to both the peritoneal and air pouch models. It has not been determined whether inhaled LPS is absorbed systemically in the lung, as it is in the peritoneum; one study found no evidence of this in humans (Hasday et al. 1999), and another study in pigs concluded it was likely to be related to the dose of LPS (Landolt et al. 2002). Comparing the pronounced plasma cytokine and leukocyte response to LPS in murine peritonitis in Chapter 3, with the absence of such a response in the ALI model, LPS is unlikely to have been absorbed systemically at the dose we administered. In a similar vein to neutrophil migration, there is substantial variation between published studies with respect to the cytokine response in BALF, with those studies involving LPS inhalation via tracheal cannulation provoking a TNFα response many orders of magnitude greater than in our study (Lorenz et al. 2001; Skerrett et al. 2004).

4.4.2.1 Platelets as mediators of neutrophil migration in ALI

Our data indicate that neutrophil migration occurs independently of platelets in LPS-induced ALI; however, platelets have been implicated in leukocyte recruitment to lung tissue in different models of acute lung injury (Pitchford et al. 2005; Zarbock et al. 2006; Kornerup et al. 2010; Grommes et al. 2012). One study demonstrated that neutrophil migration in LPS-induced ALI was dependent on platelet-neutrophil complexes (Kornerup et al. 2010); P-selectin on the surface of activated platelets has been implicated in neutrophil migration into the alveolar space in a model of allergic inflammation (Pitchford et al. 2005); and platelets are required for neutrophil migration in a model of acid-induced ALI. Evidence for the role of platelet P-selectin specifically was demonstrated in this model, through the use of bone marrow chimaeras (Zarbock et al. 2006).

Given the heterogeneity of experimental methods, a recent paper systematically investigated the role of platelets in different models of ALI (Grommes et al. 2012). In their experiments using the LPS-inhalation model of ALI, they demonstrated that depletion of platelets abrogated neutrophil migration into the alveolar space. We found the exact opposite. The paper by Grommes et al. shows that platelet-derived CCL5-
CXCL4 heteromers mediate neutrophil migration. A key step in the experimental logic is the demonstration that platelet depletion via anti-platelet serum abrogates the CCL5 response to LPS in lung tissue. Our data did not replicate this: we saw significant rises in CCL5 in the plasma and BALF of both control and platelet-depleted animals. The difference may arise from there being different mechanisms of platelet depletion between the anti-CD41 antibody we used and anti-platelet serum; no evidence was given to demonstrate the serum did not deplete additional cells. The paper also used anti-CD41 antibody, not to deplete platelets, but to block the formation of platelet-neutrophil complexes through CD41-mediated binding. They administered the anti-CD41 before LPS injection, but did not allow enough time for platelet depletion to occur. Drawing parallels with our data, the anti-CD41 antibody had no effect on neutrophil migration in these circumstances. We have no data to contradict the paper’s finding that neutrophil migration in this model is dependent on CCL5; however, our data do suggest that the CCL5 response is independent of platelets in both plasma and BALF. The possibility remains that the CCL5 response in lung tissue itself (which we did not measure) is platelet dependent, as Grommes et al. show; however, there is other evidence to show this may not be the case, as both alveolar macrophages and airway epithelium produce CCL5 during inflammation (Petrek et al. 1997; Sharma et al. 2007).

Grommes et al. (2012) used 30 min exposure to nebulised LPS (500µg/ml) from *Salmonella enteritidis* (Sigma, Munich, Germany), and lavaged the broncho-alveolar space after 4h. In contrast, we used 20 min exposure to nebulised LPS (2mg/ml) from *Escherichia coli* O127:B8 (Sigma-Aldrich, Dorset, UK), and lavaged at 6h. Male C57BL/6 were used in both cases. It should be noted that the response in control animals in the Grommes et al. study is forty-fold higher: a mean of approximately 100,000 neutrophils/ml of BALF, compared to just 2,500/ml in our mice. This higher baseline response provides much more scope for inhibition; the response in platelet-depleted mice was approximately 3,000/ml in both studies. Given the similar size of the LPS effect in platelet-depleted mice, it is the control mice that exhibit the difference between the studies. There are differences in exposure time; LPS concentration, method of administration and organism; and time of outcome measurement, which may also be responsible for the differences in this result.
Notably, our study did not show a significant cytokine response to LPS in plasma, which may indicate the small size of the inflammatory stimulus compared to other studies. There is evidence that different LPS strains exert pro-inflammatory effects to differing degrees (Netea et al. 2001). A study by Zarbock et al. used LPS (3mg/ml) from Escherichia coli O111:B4 (Sigma-Aldrich, St Louis, MO, USA) to model ALI at a 4h time point (Zarbock et al. 2006). This intervention was augmented with zymosan at 2h (a technique used to enhance pulmonary oedema in LPS-induced ALI models (Miotla et al. 1996)) and yielded approximately 8,000 neutrophils/ml in BALF, a total of the same magnitude as our findings.

Another important factor in our data was the difference in the levels of CXCL1 and CCL5 observed in the plasma of anti-CD41 treated mice compared to controls (Figure 4.5). Reductions in chemokine levels cause fewer neutrophils to be recruited to sites of inflammation (Tessier et al. 1997; García-Ramallo et al. 2002). As a result, thrombocytopaenic mice may have had a higher chemotactic stimulus driving neutrophil recruitment into the lungs. It is unclear why this should be the case, but may explain why neutrophil migration was sustained in thrombocytopaenic mice.

It is difficult to draw conclusions regarding the role of platelets in ALI from the literature. Here we showed that significant migration occurred following the removal of >90% of platelets from circulation, a clear demonstration of a neutrophil migration via a platelet-independent mechanism. There is clear evidence of the importance of platelet selectins in acid-induced ALI (Burns et al. 2001; Zarbock et al. 2006; Looney et al. 2009), yet they appear unimportant in LPS-induced ALI (Grommes et al. 2012). Hence, the importance of platelets is likely to be context dependent, varying by inflammatory stimulus.

4.4.2.2 IL-1 as a mediator of neutrophil migration in ALI

Our model of neutrophil migration in acute lung injury was independent of IL-1, a finding new to the literature. In the absence of IL-1, we observed large increases in IL-6 and TNFα and the chemokines CXCL1 and CCL5; this pro-inflammatory response was
sufficient to drive neutrophil migration independently of IL-1, and shows other cytokines to be key mediators.

Most of the research in ALI has focused on the role of TNFα as mediator of neutrophil entry in early LPS-induced ALI. Levels of TNFα peak earlier and higher than other cytokines, at a time point consistent with a role in driving neutrophil migration (Jansson et al. 2004). Although many studies and reviews implicated TNFα as the key mediator (Ware & Matthay 2000; Park et al. 2001; Goodman et al. 2003; Bhatia & Moochhala 2004), a more recent study, using TNFα deficient mice, demonstrated while some of ALI’s inflammatory characteristics were TNFα driven, neutrophil migration was independent of TNFα (Schnyder-Candrian et al. 2005). Subsequent papers have sought to explain the discord in conclusions via experiments with other molecules in the TNF system, such as TNFα converting enzyme (TACE) (Shimizu et al. 2009).

In a similar vein to TNFα, though neutrophil recruitment is independent of IL-1, the IL-1 system has been implicated in the resolution of LPS-induced acute lung injury. In one study that investigated LPS-induced ALI over 72h, IL-1β activity peaked 48h after LPS exposure (Herold et al. 2011). Macrophage-derived IL-1Ra was been shown to attenuate broncho-alveolar inflammation and airway epithelial damage 72h after LPS in bone marrow chimaeras. This shows that the IL-1 responses we observed in BALF may not drive neutrophil infiltration, but could have a later role in the inflammatory process. A key cytokine driver of neutrophil migration in LPS-induced ALI remains elusive; however, IL-1 is implicated in mediating neutrophil migration in sterile inflammation of the lung, which reflects the situation in peritoneal and air pouch models (Kuipers et al. 2012).

4.4.3 Mediation of neutrophil migration in vascular beds of different tissues
We have discussed previously the potential for different mechanisms to facilitate neutrophil migration across the vascular beds of different tissues. In Chapters 3 and 4, we demonstrated, via the use of knockout mice, that IL-1 was not required during the migration of neutrophils into the inflamed peritoneum, air pouch and broncho-alveolar space. In the first two models, depletion of platelets via anti-CD41 antibody abrogated
neutrophil migration, indicating that such migration was dependent on platelets. In ALI, however, depletion of platelets had no effect on the migration of neutrophils into the broncho-alveolar space. Given the consistency of our approach across different models, it is possible to compare between them. There is a difference, in our hands, in the requirement for platelets to facilitate neutrophil migration in the lungs, compared to the peritoneum and air pouch. This would support the general hypothesis that different vascular beds exhibit different inflammatory pathways to mediate neutrophil migration. There is evidence that the lungs represent a unique environment for neutrophil migration; the lungs sequester more leukocytes in a non-inflammatory state, and many of the lungs specialised cells having a demonstrable effect on migrating neutrophils (Downey et al. 1993; Walker et al. 1995; Behzad et al. 1996; Doerschuk 2001).

The primary motivation for using air pouch and ALI models was to determine if the IL-1-independent nature of neutrophil migration to the peritoneum was common to other extra-cerebral tissues. In all the tissues tested, IL-1 was not required. Although it is not possible to conclude that IL-1 is dispensable for driving migration to all extra-cerebral tissues, these data strongly support a general IL-1-independent mechanism across multiple tissues outside the brain. In vitro, IL-1 is a proven mediator of cerebrovascular inflammation, and a facilitator of neutrophil migration across cultured MBECs (Thornton et al. 2010). This may suggest different mechanisms drive neutrophils to enter brain tissue in comparison to extra-cerebral tissues, which has not been definitively tested in vivo.

4.5 Conclusion

A reproducible model of LPS-induced air pouch inflammation was characterised for the measurement of neutrophil migration. The migration of neutrophils into a dorsal air pouch was demonstrated to be dependent on platelets and independent of IL-1. The migration of neutrophils into the broncho-alveolar space, following LPS-induced ALI, was independent of both platelets and IL-1.

The data in this chapter suggest a common IL-1-independent mechanism in mediating neutrophil migration across the vascular beds of extra-cerebral tissues in vivo, and to
support the concept of tissue-specific mechanisms. Further studies are required to confirm the role of IL-1 in mediating cerebrovascular neutrophil migration *in vivo*, and to provide further evidence of tissue-specific mechanisms.
Chapter 5

Contribution of platelets and IL-1 to neutrophil migration in LPS-induced cerebral inflammation
5.1 Introduction

Data in previous chapters indicate that neutrophil migration to inflamed extra-cerebral tissues in vivo occurs via an IL-1 independent mechanism, which was common to all tissues tested. In contrast, there were differential requirements for platelets because LPS-induced neutrophil migration to the peritoneum and air pouch required platelets whereas migration to the lung was platelet-independent. The apparent lack of requirement for IL-1 in driving inflammation in multiple tissues outside the brain was unexpected and contrasts with recent in vitro evidence (Thornton et al. 2010), supporting platelet-derived IL-1α as a key driver of cerebrovascular inflammation. This suggests that the cerebrovasculature may have different molecular requirements for driving neutrophil migration into the brain compared to vascular systems outside the brain and that further evaluation in an in vivo brain inflammation model is warranted.

In this chapter, the studies aim to determine if neutrophil migration into the brain parenchyma following LPS injection is dependent on platelets and IL-1. We use a model involving direct intracerebral LPS injection.

Many studies have reported the effects of LPS injection into the mouse brain. Notably, Andersson et al. (1992) compared the inflammatory response to LPS in the CNS to that in the peritoneum and skin. The CNS required larger doses of LPS to induce inflammatory cell recruitment. The authors characterised the inflammatory response into three phases. The first of these involved relatively limited neutrophilic infiltrate peaking 12-24h post-injection, with small numbers adherent to the cerebrovasculature. Based on this, we performed initial characterisation at a 24h time point, and subsequently also performed 6h experiments to investigate cytokine mediators that might drive neutrophil accumulation.

A previous study investigating the CNS response to LPS used Hamilton syringes to administer intracerebral injections. The investigators noted LPS leakage up the cannula track, resulting in florid immune cell recruitment in the cerebroventricular system, notably including the choroid plexuses (Andersson et al. 1992). Following data evaluating the effect of different methods of intracerebral administration, we used glass
micro-needles in an effort to minimise the diameter of the cannula track. Glass micro-
needles have been shown to exhibit minimal injection artefact, as evaluated by extent of
mechanical injury, blood-brain barrier breakdown, and neutrophil recruitment
(McCluskey et al. 2008).

The aims of the studies in this chapter were to determine:

1. if platelets are required to mediate neutrophil migration into the brain parenchyma;
2. if IL-1 is required to mediate neutrophil migration into the brain parenchyma;

5.2 Methods and materials

5.2.1 General methods and materials

A pilot experiment was conducted first to determine the effect size and variation for
power analyses of LPS in stimulating neutrophil migration into brain tissue, we injected
the striatum of two groups of C57BL/6J mice with vehicle (n=3) or LPS (1µl of 4mg/
ml, n=3) to induce inflammation.

Four groups of C57BL/6J mice (n=5) were used for each of the experiments outlined
below, based on power analyses from pilot experiments (Appendix 7.5). Groups were
randomised and blinded to investigators throughout treatment and analysis. For all
experiments (except those involving brain tissue homogenates), brains were removed
and processed as described in Chapter 2, and underwent immunohistochemistry for
neutrophil quantification. The counting map is shown in Figure 2.2, Chapter 2 and
reiterated below; three fields ipsilateral to the LPS injection were quantified per mouse
at the areas represented by yellow squares.
Neutrophils were quantified using a 10x10mm graticule at 20x magnification, and by a cumulative total of the three areas surveyed. Corresponding contralateral fields were also quantified, to demonstrate the loco-regional specificity of LPS. Neutrophils and leukocytes in cardiac blood were quantified via flow cytometry. Cytokine concentration in cardiac blood were quantified with multiplex analysis using cytometric bead array or ELISA.

### 5.2.2 Contribution of platelets to neutrophil migration during cerebral inflammation

To determine whether neutrophil migration is platelet-dependent in a model of cerebral inflammation (see 2.2.4), mice were treated with anti-CD41 antibody or IgG control as described previously, and the striatum injected after 24h with LPS or vehicle. Two separate experiments were performed at different time points: 6 and 24h after injection with LPS or vehicle.

A further experiment at a 6h time point was conducted to collect brain tissue for cytokine quantification using CBA.

We used a second experimental strategy involving anti-Gplb antibody to determine the role of platelets; this antibody inhibits platelet adhesion to the blood vessel wall, rather than depleting platelet numbers as with anti-CD41. Mice were injected i.v. in the tail vein with anti-Gplb antibody or IgG isotype control 4h before intrastriatal injection of LPS or vehicle.
Mice were culled at indicated time points, and their brains were removed for immunohistochemical analysis, including neutrophil quantification. Circulating platelet numbers were measured via Coulter Counter and flow cytometric analysis of cardiac blood samples.

5.2.3 Contribution of IL-1 to neutrophil migration during cerebral inflammation

To determine whether neutrophil migration is IL-1-dependent in cerebral inflammation, the striata of wild-type C57BL/6J and IL-1α/β−/− double knockout mice were injected with LPS or vehicle. Again, two separate experiments were performed at different time points: 6 and 24h after injection with LPS and vehicle. Another experiment was conducted to determine the specific role of the IL-1α ligand, using IL-1α−/− single ligand knockout mice.

5.2.4 Contribution of systemic IL-1α to neutrophil migration during cerebral inflammation

To determine whether neutrophil migration could be blocked with systemic anti-IL-1 interventions, we used IL-1Ra and antibodies to both IL-1 ligands. In the first experiment, IL-1Ra (10mg/kg) or saline was administered i.p. 1h before and 2h after LPS injection. Mice were culled and samples taken 24h after injection of LPS.

In a second experiment, mice were injected i.p. with anti-IL-1α (1mg/kg), anti-IL-1β (4mg/kg) or IgG isotype control (4mg/kg), 24h before intrastriatal injection of LPS. Dosages of antibody were based on the manufacturer’s 50% neutralisation dose (ND50), and previous data on cytokine responses to LPS. Mice were culled and samples collected 24h after injection of LPS.
5.3 Results

5.3.1 LPS-induced neutrophil migration to the brain is dependent on platelets

To determine whether neutrophil migration was dependent on platelets in response to LPS-induced cerebral inflammation, we treated C57BL/6J mice with anti-CD41 antibody or IgG isotype control, 24h before intrastriatal injection of LPS. LPS induced a significant increase in the number of neutrophils migrating into the cerebral parenchyma of control mice at 24h (p<0.01, Figure 5.1a). Anti-CD41 antibody depleted >80% of circulating platelets in treated animals compared to IgG-injected controls (p<0.001, Figure 5.1b). Platelet depletion with anti-CD41 antibody significantly reduced the number of migrated neutrophils in cerebral tissue in response to LPS injection (p<0.05, Figure 5.1a,d). These data show that neutrophil migration in the brain, in response to LPS injection, is dependent on platelets in circulation. Platelets formed aggregates, and co-localised with inflamed blood vessels expressing VCAM-1, in LPS treated mice (Figure 5.1c). In thrombocytopenic mice treated with LPS, blood vessels expressed VCAM-1, which did not co-localise with platelets (Figure 5.1d). At 6 and 24h, IL-1α was present in the striatum of LPS injected mice and co-localised with Iba1-positive microglia (Figure 5.1e).

The distribution of migrated neutrophils in response to LPS in the brain parenchyma was concentrated on the injection site. There was a greater spread posteriorly into the cortex than anteriorly, though there was no evidence from monastral blue staining, observed during brain slicing, that the injection site was not level with bregma coronally. Few neutrophils were found in the striatum of the contralateral hemisphere. There was dense neutrophil recruitment to the choroid plexus in the ventricles on both sides of the midline in response to LPS (see also Appendix 7.5).
Figure 5.1 - Neutrophil migration in LPS-induced cerebral inflammation is dependent on platelet recruitment. Mice were injected with anti-CD41 antibody or IgG control 24h before inflammation was induced via intrastriatal injection of LPS. Brains were harvested at 24h after LPS injection. Representative immunofluorescence for VCAM-1 and CD41 showing platelet adherence and aggregates in IgG + LPS treated mice. Data are mean ±sd. *p<0.05, **p<0.01. Alexa Fluor 594 labeled IgG and Alexa Fluor 488 labeled anti-CD41 antibody were used. Neutrophils were enumerated in brain tissue slides by counting the number of neutrophils adherent to VCAM-1 and CD41 positive platelets.
5.3.2 Blood neutrophil and cytokine responses to LPS-induced cerebral inflammation and effects of platelet depletion

At 6h following LPS injection, no neutrophils migrated into brain tissue, however there were significant changes to circulating leukocytes and plasma cytokines, which we compared to the 24h time point. In response to LPS injection, there was an increase in the number of circulating neutrophils in IgG treated mice at 24h (p<0.01, Figure 5.2). At 24h, there was a significant increase in circulating neutrophil numbers in response to LPS in anti-CD41 treated mice (p<0.05, Figure 5.2). LPS treatment resulted in a significant decrease in circulating total leukocytes in IgG treated mice at both 6 and 24h (p<0.05, Figure 5.2).

Significant increases in plasma levels of IL-1α, IL-6, TNFα, CXCL1 and CCL5 were observed in response to LPS at 6h (p<0.01, Figure 5.2); these increases had attenuated by 24h, with only CCL5 remaining significantly increased (p<0.001, Figure 5.2). Plasma IL-1ß showed no response to LPS at either time point. Treatment with anti-CD41 antibody significantly abrogated the effect of LPS on IL-1α, IL-6, TNFα, CXCL1 and CCL5 levels (p<0.05, Figure 5.2).
Figure 5.2 - Cytokine profiles in plasma at 6 and 24h in response to LPS-induced cerebral inflammation.
Mice were injected with anti-CD41 antibody or IgG control 24h before inflammation was induced by intrastriatal injection of LPS. Cardiac blood was sampled 6 or 24h after LPS. Cytokines were quantified in plasma using CBA. Data are mean ±sd. *p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction.
5.3.3 Cerebral cytokine and chemokine responses to LPS-induced cerebral inflammation and effects of platelet depletion

To examine potential mediators that drive neutrophil influx, we collected brains for homogenisation at 6h following LPS injection. LPS induced significant increases in the levels of IL-1α, IL-β, IL-6, TNFα, CXCL1 and CCL5 in the ipsilateral cerebral hemisphere (p<0.05, Figure 5.3). Mice that underwent platelet depletion by anti-CD41 antibody showed no differences compared to controls, with increases remaining significant in IL-1α, IL-β, IL-6 and CCL5 (p<0.01), and showing non-significant increases in TNFα and CXCL1 (Figure 5.3).

Figure 5.3 - Cytokine profiles in brain tissue in response to LPS-induced cerebral inflammation.
Mice were injected with anti-CD41 antibody or IgG control 24h before inflammation was induced via intrastrital injection of LPS. Brains were harvested 6h after LPS. Cytokines were quantified in tissue homogenates of the hemisphere ipsilateral to the injection by CBA. Data are mean ±sd. *p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction.
5.3.4 Anti-GpIb inhibition attenuates LPS-induced neutrophil migration

To further examine if platelet function is important for neutrophil migration in response to LPS-induced cerebral inflammation, we treated C57BL/6J mice with anti-GpIb antibody or IgG isotype control, 4h before intrastriatal injection of LPS. Following LPS injection, a significant number of neutrophils migrated into the cerebral parenchyma of control mice at 24h (p<0.001, Figures 5.4a, 5.5). In addition, immunofluorescence revealed more neutrophils co-localising with inflamed blood vessels expressing VCAM-1 (Figure 5.5). Anti-GpIb antibody had no effect on numbers of circulating platelets in compared to IgG-injected controls (Figure 5.1b). Anti-GpIb antibody significantly reduced the number of neutrophils in brain tissue after LPS (p<0.01, Figure 5.4a).

Figure 5.4 - Neutrophil migration into brain tissue in LPS-induced inflammation is blocked by anti-GpIb antibody.
Mice were injected with anti-GpIb antibody or IgG control 4h before inflammation was induced by intrastriatal injection of LPS. Brains were harvested at 24h after LPS injection. (a) Neutrophils were quantified in brain tissue with immunohistochemistry for SJC4. (b) Platelets, (c) neutrophils and leukocytes were quantified in cardiac blood with flow cytometric analysis. Data are mean ±sd. **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction.
These data show that neutrophil migration in the brain, following LPS injection, is partially driven by GpIb function on platelets in circulation. In this experiment, LPS had no effect on circulating neutrophil levels in either anti-GpIb antibody treated mice or control animals. LPS injection was associated with non-significant decreases in total circulating leukocytes in both sets of animals (Figure 5.4c).

Platelets formed aggregates, and co-localised with inflamed blood vessels expressing VCAM-1, in IgG and LPS treated mice as previously seen (Figure 5.5). In mice treated with anti-GpIb and LPS, blood vessels expressed VCAM-1, but did not co-localise with platelets (Figure 5.5).
**Figure 5.5 - Neutrophil migration and platelet aggregation in LPS-induced cerebral inflammation is inhibited by anti-GpIb antibody.**

Mice were injected with anti-GpIb antibody or IgG control 24h before inflammation was induced by intrastriatal injection of LPS. Brains were harvested at 24h after LPS injection. Immunofluorescence staining for (a) neutrophils (SJC4), (b) VCAM-1 and platelets (CD41) and (c) VCAM-1 and neutrophils (SJC4) in brain tissue from IgG and LPS, and anti-GpIb and LPS injected mice. Images are representative of treatment groups.
5.3.5 Contribution of IL-1 to neutrophil migration in response to LPS-induced cerebral inflammation

To determine the role of IL-1 in mediating neutrophil migration, we injected LPS intrastriatally into wild-type (C57BL/6J) and IL-1α/β-/- double knockout mice. Neutrophil migration in WT mice injected with LPS showed a significant increase over vehicle injected controls at 24h (p<0.001, Figure 5.6a,b), replicating previous data. At 6h, no neutrophils had migrated in response to LPS. The number of neutrophils that had migrated into brain tissue after LPS injection was significantly reduced in IL-1α/β-/- compared to WT animals (p<0.001, Figure 5.6a). These data show that neutrophil migration in response to LPS-induced cerebral inflammation requires IL-1 as a mediator. At 6 and 24h, IL-1α was induced by LPS in WT mice and co-localised with Iba1-positive microglia, while no IL-1α was detected in IL-1α/β-/- mice (Figure 5.6c). WT and IL-1α/β-/- injected with LPS both exhibited inflamed blood vessels expressing VCAM-1 at 6 and 24h, and platelets co-localising with VCAM-1 were detected in both groups (Figure 5.6d).
Figure 5.6 - Neutrophil migration in LPS-induced cerebral inflammation is dependent on IL-1

Wild-type or IL-1α/β−/− double knockout mice were injected intrastratally with LPS. Brains were harvested at 6 and 24h after LPS injection. (a,b) Neutrophils were quantified in brain tissue via immunohistochemistry for SJC4. Data are mean ±sd. *p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction. Representative immunofluorescence for (c) Iba1 and IL-1α and (d) VCAM-1 and CD41 in each treatment group.
Significant increases in plasma levels of IL-1α, IL-6, TNFα, CXCL1 and CCL5 were observed in response to LPS in control animals at 6h (p<0.05, Figure 5.7). Plasma IL-1β showed no significant response to LPS. This pattern was previously observed in 5.3.2. As expected, IL-1α/β−/− did not show an IL-1 response to LPS. There were no significant differences in the cytokine response to LPS between knockout mice and control animals, though the LPS-induced increase in TNFα and IL-6 did not reach significance in knockout mice as they had in WT controls.
Figure 5.7 - Cytokine profiles in plasma in response to LPS-induced cerebral inflammation in wild-type and IL-1\(\alpha\)/\(\beta\)\(^{-/-}\) double knockout mice.

Wild-type and IL-1\(\alpha\)/\(\beta\)\(^{-/-}\) double knockout mice were injected intrastratally with LPS or vehicle. Cardiac blood was sampled 6h after LPS injection. Cytokines were quantified in plasma by CBA. Data are mean ±sd. *p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction.
5.3.6 Contribution of IL-1α to neutrophil migration in response to LPS-induced cerebral inflammation

To determine the role of IL-1α in mediating neutrophil migration, we injected LPS into the striatum of wild-type (C57BL/6J) and IL-1α⁻/⁻ single ligand knockout mice. Neutrophil migration in WT mice following LPS injection showed a significant increase over vehicle injected controls at 24h (p<0.001, Figure 5.8a), replicating the pattern observed in previous data. The number of neutrophils that had migrated into brain tissue after LPS injection was significantly reduced in IL-1α⁻/⁻ compared to WT animals (p<0.001, Figure 5.8a). These data show that neutrophil migration in LPS-induced cerebral inflammation is mediated by IL-1α. The magnitude of the effect was similar to that observed in IL-1α/β⁻/⁻ knockout mice, suggesting IL-1α may play the predominant role in this process. No IL-1α was detected in the striatum of mice injected with PBS. In response to LPS, IL-1α was detected, and co-localised, with Iba1-positive microglia in WT animals. There was no IL-1α detected in IL-1α⁻/⁻ mice (Figure 5.8b).

At 24h, we have previously demonstrated attenuated plasma cytokine responses to LPS, and the pattern was replicated here; at 24h in this experiment, there were significant increases in only TNFα and CCL5 in both WT and IL-1α⁻/⁻ injected with LPS, over vehicle injected controls (p<0.01, Figure 5.8c). We did not undertake a 6h time point analysis of cytokines with IL-1α⁻/⁻ single knockout mice. WT mice injected with LPS exhibited a greater density of inflamed blood vessels expressing VCAM-1 at 24h than their IL-1α⁻/⁻ counterparts, and platelets co-localising with VCAM-1 were detected in both groups (Figure 5.8d).
Figure 5.8 - Neutrophil migration in LPS-induced cerebral inflammation is dependent on IL-1α
Wild-type or IL-1α−/− double knockout mice were injected intrastriatally with LPS. Brains were harvested and cardiac blood samples taken 24h after LPS injection. (a) Neutrophils were quantified in brain tissue with immunohistochemistry for SJC4. (b) Representative immunofluorescence for IL-1α and Iba1 in each treatment group. (c) Cytokines in plasma were quantified using CBA. Data are mean ±sd. *p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction. (d) Representative immunofluorescence for VCAM-1 and CD41 in each treatment group.
5.3.7 Neutralisation of systemic IL-1 action has no effect on neutrophil migration in response to LPS-induced cerebral inflammation

Experiments above indicated an important role for IL-1, particularly IL-1α, in driving neutrophil migration to the brain in response to intracerebral LPS. Deficiency of both IL-1 agonists or IL-1α alone had no effect on systemic inflammatory profiles suggesting that IL-1 actions within the brain may be most important. To gain further insight to whether IL-1 action in the brain and/or systemically predominate we tested the effects of the systemic anti-IL-1 interventions, IL-1Ra and anti-IL-1 antibodies.

To determine whether neutrophil migration could be blocked by systemically administered IL-1Ra, we injected C57BL/6J mice i.p. with IL-1Ra 1h before and 2h after intrastriatal injection of LPS. Following injection of LPS, a significant number of neutrophils migrated into the cerebral parenchyma of control mice at 24h (p<0.01, Figures 5.9a). IL-1Ra had no effect on numbers of migrated neutrophils in treated animals compared to placebo-injected controls (Figure 5.9a). These data show that neutrophil migration in the brain, following LPS injection, can not be blocked by the systemic administration of IL-1Ra. In this experiment, LPS injection significantly decreased total circulating leukocytes in both groups of animals (p<0.05, Figure 5.9b).

There were significant increases following LPS in plasma TNFα, CXCL1 and CCL5 in vehicle-treated mice at 24h (p<0.05, Figure 5.9c). There were significant increases following LPS in plasma IL-6 TNFα, CXCL1 and CCL5 in animals treated with IL-1Ra (p<0.05, Figure 5.9c). There was no change in the level of either IL-1 ligand in response to LPS in both groups. The plasma CCL5 level in IL-1Ra/LPS injected mice was significantly higher than in vehicle-treated controls (p<0.001, Figure 5.9c).
Figure 5.9 - IL-1Ra has no effect on neutrophil migration in LPS-induced cerebral inflammation
Mice were injected intrastriatally with LPS and received either IL-1Ra or placebo. Brains were harvested and cardiac blood samples taken 24h after LPS injection. (a) Neutrophils were quantified in brain tissue through immunohistochemistry for SJC4. (b) Circulating leukocytes were quantified via flow cytometry. (c) Cytokines in plasma were quantified with multiplex analysis with cytometric bead array. Data are mean ±sd. *p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction.
To determine whether neutrophil migration could be blocked by systemically administered antibodies to either IL-1 ligand, we injected C57BL/6J mice i.p. with anti-IL-1α, anti-IL-1β or IgG control antibodies 24h before intrastriatal injection of LPS. Following injection of LPS, a significant number of neutrophils migrated into the cerebral parenchyma of control mice at 24h (p<0.05, Figures 5.10a). Neither anti-IL-1α nor anti-IL-1β antibody had an effect on numbers of migrated neutrophils in treated animals compared to placebo-injected controls (Figure 5.10a); however the number of neutrophils migrating into LPS/anti-IL-1β injected mice was not significantly elevated over their PBS injected controls. No rat IgG was detected in brain tissue by immunohistochemistry, which would indicate the antibodies did not cross the BBB (Appendix 7.8). These data show that neutrophil migration in the brain, following LPS injection, could not be blocked by the systemic administration of antibodies to either IL-1 ligand. LPS injection significantly had no effect on total circulating leukocytes in any of the three treatment groups (Figure 5.10b). There were non-significant increases following LPS in plasma IL-6, CXCL1 and CCL5 in all three treatment groups at 24h (Figure 5.10c). There was no change in the level of IL-1α, IL-1β or TNFα in response to LPS in any group.
**Figure 5.10 - Anti-IL-1α and anti-IL-1β antibodies have no effect on neutrophil migration in LPS-induced cerebral inflammation**

Mice were injected intrastriatally with LPS and received either anti-IL-1α, anti-IL-1β or IgG control antibody. Brains were harvested and cardiac blood samples taken 24h after LPS injection. (a) Neutrophils were quantified in brain tissue by immunohistochemistry for SJC4. (b) Circulating leukocytes were quantified by flow cytometry. (c) Cytokines in plasma were quantified via CBA. Data are mean ±sd. *p<0.05, **p<0.01, ***p<0.001 via one-way ANOVA with post-hoc Bonferroni correction.
5.4 Discussion

In this chapter, we characterised an LPS-induced cerebral inflammation model as a tool to study neutrophil migration in the brain. Using this model, we demonstrated that neutrophil migration into the brain parenchyma required the presence of platelets in circulation, and was dependent on IL-1α. The systemic anti-IL-1 interventions IL-1Ra and antibodies to both IL-1 ligands failed to inhibit neutrophil migration.

5.4.1 Neutrophil migration in LPS-induced cerebral inflammation

Throughout the experiments in this chapter, we used an in vivo model of cerebral neutrophil migration induced by intrastriatal LPS injection. We observed a consistent neutrophil response to intrastriatal LPS across the different experiments in this chapter. Using a reproducible quantification strategy, we recorded in different experiments a consistent number of neutrophils (approximately 400-600) in counting sites of brain tissue at 24h in response to LPS in control animals, with no neutrophils at 6h. A response of this magnitude has been previously observed following the injection of similar doses of LPS directly into the brain parenchyma (Andersson et al. 1992; Blond et al. 2002).

The change in the number of total leukocytes in circulation in response to LPS varied between our experiments. There was a trend across experiments for LPS injection to induce a decrease in circulating leukocytes and an increase in circulating neutrophils, which we observed at 6 and 24h following LPS injection. This effect was not detected in the anti-IL-1 antibody study, yet there is evidence that LPS was active, as neutrophils were recruited to brain tissue in control mice. As explained in previous chapters, the neutrophil effect may result from increased release from the bone marrow or marginating pool, and the leukocyte effect from circulating leukocyte adherence to the vascular endothelium and the redistribution of lymphocytes to lymph nodes following injection of LPS.

We examined the cytokine response in brain tissue 6h after LPS injection. There was a significant response in IL-1α, IL-1β, IL-6, TNFα and the chemokines CXCL1 and CCL5. The short-term increases in IL-1β and TNFα have been observed before.
et al. 2002). This study also described attenuation of the cytokine response by 24h; although we did not measure cytokines in brain tissue at this time point, the cytokine peak in plasma we saw at 6h had attenuated by 24h. This appears consistent with the findings of Blond et al. (2002).

One study qualitatively examined the inflammatory response to 2µg of LPS injected into the mouse hippocampus (Andersson et al. 1992). They observed neutrophils present in the brain parenchyma 12h after injection, with a peak between 12-24h. The authors also noted that there were neutrophils present in the ventricular system and choroid plexus; these cells were also present in our study. The authors speculate that LPS may have leaked into the ventricular system up the needle track; however, the study used a Hamilton micropipette to perform injections, which is associated with a wider injection track and a greater inflammatory response than the glass microneedles that we used (McCluskey et al. 2008). The authors also propose that LPS may reach the choroid plexus by diffusion through the extracellular space and across the ependyma, the cellular layer that lines the ventricular system.

The temporal pattern of cerebrovascular inflammation has been investigated previously (Bell & Perry 1995). An increase in VCAM-1 expression on cerebral blood vessels was the most sensitive and specific response to LPS, showing large increases in expression, and with ICAM-1 and PECAM being detectable on unstimulated endothelium. We observed VCAM-1 expression 6h following LPS injection, which was maintained at 24h, consistent with previous findings (Engelhardt et al. 1994; Bell & Perry 1995).

5.4.2 Platelets as mediators of neutrophil migration in cerebral inflammation

In this chapter, we demonstrate that neutrophil migration in LPS-induced cerebral inflammation is dependent on the presence of platelets in circulation, and the function of the platelet surface adhesion receptor GpIb. Both anti-CD41 antibody and anti-GpIb antibody caused a similar reduction in the number of neutrophils present in brain tissue at 24h, indicating that the mechanism by which platelets facilitate neutrophil migration is mediated through the action of the GpIb adhesion receptor. We could not evaluate the temporal relationship between platelet adhesion and neutrophil migration because we
sampled only at 6 and 24h; however, a previous study has demonstrated that platelet rolling occurs before leukocyte adhesion (Carvalho-Tavares et al. 2000). Importantly, the anti-CD41 antibody replicated the degree of platelet depletion seen in Chapters 3 and 4, while the anti-GpIb antibody had no effect on platelet numbers. The antibody prevented the adhesion of platelets to VCAM-positive blood vessels in the brain at 24h. One study of experimental stroke found that neutralisation of GpIb reduced brain damage at 24h (Kleinschnitz et al. 2007). While the paper did not look at neutrophil migration, it does implicate platelet adhesion to brain endothelium as an important factor in the evolution of brain injury, a hypothesis consistent with our data.

One study made use of spinning-disc confocal microscopy to characterise in vivo the interactions between platelets, leukocytes and brain endothelium (Jenne et al. 2011). In control animals, there were very few interactions between platelets and the endothelium, and there was no platelet aggregation. 4h after i.v. injection of LPS, platelets began to adhere to the endothelium, and also to adhere to adherent neutrophils; at later time points there was increased platelet and neutrophil adherence. The experiments also showed platelet-neutrophil aggregates free in circulation. The authors emphasised the dynamic nature of these interactions, with platelets forming unstable aggregates, and “sloughing off” downstream in the circulation. We found very few adherent platelets at 6h following intrastriatal LPS injection, with a large number co-localising with VCAM-positive vessels at 24h. The difference in findings at the 6h time point may be explained by the use of i.v. LPS by Jenne et al. (2011), which would have induced platelet activation at earlier time points than intrastriatal infusion. Leukocyte adherence has also been studied in the brain in response to ischaemia (Granger et al. 1989). Adherent leukocytes were observed 10 min after ischaemia onset, and the number of adherent neutrophils was directly proportional to extravasated neutrophils.

Thornton et al. (2010) demonstrated that activated platelet-derived conditioned medium was sufficient to mediate expression of ICAM-1 and release of CXCL1 by MBECs in vitro. Conditioned medium was also sufficient to drive neutrophil migration across cultured MBEC monolayers. This indicated that direct platelet-endothelial cell-cell contact may not be necessary for endothelial activation. Our experiment with the GpIb
antibody does not appear consistent with this hypothesis; however, it may be that adhesion via GpIb plays a role in platelet activation, and that its inhibition abrogates the platelet release of cytokines. Our data show that VCAM-1 is expressed by cerebral blood vessels at 6h, before we observed platelet adherence. This is consistent with a mechanism independent of platelet-endothelial interactions driving the activation of cerebrovascular inflammation; however, it remains a possibility that a platelet mediator, such as IL-1α, released into plasma may drive the early blood vessel VCAM-1 response.

Platelet depletion via anti-CD41 antibody abrogated the 6h LPS-induced increase in plasma IL-1α in the cerebral model, as previously demonstrated in other in vivo models in Chapters 3 and 4. This suggests that the plasma IL-1α released in response to LPS is derived from, or triggered by, platelets. In the cerebral model, the effect of platelet depletion on plasma cytokines was more widespread than in other models, inducing significant reductions in IL-6, CXCL1 and CCL5 in addition to IL-1α. Anti-CD41 antibody had no effect on the level of IL-1α induced at 6h by LPS in brain homogenate. This replicates a pattern observed previously in the air pouch model of inflammation. While platelets are necessary for the IL-1α response in plasma, there must be another source for IL-1α in brain tissue. Microglia are a good candidate for the cellular source of this IL-1α; following cerebral ischaemia, IL-1α (not IL-1β) is expressed by microglia (Luheshi et al. 2011). In this way, microglia may play a similar role to air pouch macrophages in producing early IL-1α in response to LPS. This is consistent with microglial derivation from a myeloid lineage (Ginhoux et al. 2010).

5.4.3 IL-1 as a mediator of neutrophil migration in cerebral inflammation

IL-1α is released by platelets and has been shown to mediate cerebrovascular inflammation in vitro (Thornton et al. 2010). To determine the role of IL-1 in mediating neutrophil migration in vivo following LPS-induced cerebral inflammation, we used IL-1α/β−/− double knockout and IL-1α−/− single ligand knockout mice. Neutrophil migration was significantly reduced in both strains of mouse, demonstrating that IL-1α is important for this process to occur in vivo. This is in keeping with the findings of a recent study, which demonstrated reduced neutrophil migration into the brain following
experimental stroke in IL-1α/β-/- double knockout mice (Allen et al. 2012). Another study demonstrated the requirement of IL-1R1 for neutrophil recruitment following a variety of inflammatory stimuli (Ching et al. 2005). The study by Allen et al. (2012) also observed a reduction in the expression of VCAM-1 after stroke in IL-1α/β-/- mice; in contrast, we saw similar levels of VCAM-1 expression following LPS injection in WT and knockout mice. TNFα is capable of inducing brain endothelial CAM expression independently, though to a lesser degree than IL-1 (Henninger et al. 1997; Ching et al. 2005; 2007; Alexander et al. 2008), and our data show that TNFα is significantly increased after LPS in knockout mice. This may explain the equivalence in CAM expression we observed in mice lacking IL-1. Platelets were adherent to VCAM-positive blood vessels in the brain at 24h, but not at 6h, which we previously observed.

We examined the 6h plasma cytokine profiles of IL-1α/β-/- double knockout mice in response to LPS. The LPS-induced increase in levels of IL-6 and TNFα in these mice was lower than in WT mice, though it did not reach significance. Both cytokines, but IL-6 in particular, may be expressed downstream of IL-1 signalling; we may therefore expect this cytokine profile in mice lacking both IL-1 ligands. There was no difference in the level of the chemokines CXCL1 and CCL5, which is important as these may be mediators that do not rely on IL-1 for their production. It has been noted that with IL-1 single ligand knockout mice, inflammatory responses are compensated for by the remaining IL-1 ligand (Boutin et al. 2001). We saw no evidence of this in the IL-1α-/- single knockouts at 24h, though by this time point the cytokine response to LPS has largely attenuated. Indeed, given the similar effect sizes of single and double IL-1 ligand knockout, our data may indicate a lack of compensation.

5.4.4 CNS-derived IL-1α and platelets independently mediate neutrophil migration in cerebral inflammation

In vitro IL-1Ra interventions abrogated the effects of conditioned medium, derived from activated platelets, on neutrophil migration across cultured MBEC monolayers. In a similar in vitro experiment, anti-IL-1α (and not anti-IL-1β) antibody prevented the expression of CAMs and release of CXCL1 by MBECs. In vivo, systemic administration of IL-1Ra and anti-IL-1α antibody had no effect on neutrophil migration
It is possible that the doses of each intervention was not sufficient to affect IL-1 function. IL-1Ra was administered in a dose previously demonstrated as efficacious in cerebral ischaemia (Pradillo et al. 2012). The dose of anti-IL-1α given was a tenth of the dose given in a recent paper in which it was effective in blocking neutrophil recruitment to sterile peritonitis (Chen et al. 2007), however we calculated its dose specifically for this model based on previous data and the manufacturer’s stated ND50. As with our previous experience at the 24h time point in this model, the level of LPS-induced plasma cytokines had attenuated in these experiments. The only notable difference between IL-1Ra treated animals and controls was a significantly higher level of plasma CCL5 at 24h, though it is unclear why this may be the case. No rat IgG was detected in brain tissue following i.p. injection of the rat anti-mouse anti-IL-1 antibodies. No detectable IL-1Ra reached the brain parenchyma at the site of LPS injection, just as mouse IgG, when detected in brain slices, which would normally indicate BBB breakdown, predominated only in periventricular areas (Appendix 7.6). Previous experiments investigating the pharmacokinetics of systemically-administered IL-1Ra in rats have shown that, although it reaches the CSF, it only penetrates the parenchyma in areas of BBB breakdown (Greenhalgh et al. 2010).

Though the antibody study showed no difference between treated animals and controls, there was a non-significant decrease in LPS-induced neutrophil migration in mice injected with anti-IL-1β. There were no other differences that we observed between the groups that may explain this difference. There was also a large number of neutrophils in the brain tissue of mice who received anti-IL-1β antibody and an intrastriatal injection of vehicle; this was not caused by a single high responder, but by two animals, and there were no experimental factors that could lead to their exclusion, nor did these animals exhibit high responses in any other of the outcomes measured. Though the effect of anti-IL-1β antibody remains equivocal in these data, the data from the IL-1Ra intervention support the conclusion that IL-1 in circulation does not affect neutrophil migration into brain parenchyma.

Neither systemic anti-IL-1 intervention affected neutrophil migration in the brain. We had already demonstrated that platelet depletion and genetic knockout of IL-1α both
abrogated neutrophil migration. Following platelet depletion, the increase in plasma IL-1α following LPS is abolished, yet IL-1α expression in the brain is maintained; indicating that platelets are responsible for the plasma IL-1α response to LPS, but not the cerebral IL-1α response. Plasma IL-1α is not essential, and is unlikely to be responsible for driving neutrophil migration in this model, as its effects would have been neutralised by IL-1Ra or anti-IL-1α antibody. Hence it is probable that CNS-derived IL-1α that drives neutrophil migration; and therefore, IL-1α and platelets mediate neutrophil migration in cerebral inflammation independently of each other.

5.5 Conclusion
A reproducible model of LPS-induced cerebral inflammation was characterised for the measurement of neutrophil migration. The migration of neutrophils into the brain parenchyma was demonstrated to be dependent on platelets, through platelet depletion with anti-CD41 antibody; and dependent on IL-1α, in experiments with IL-1α/β−/− double ligand and IL-1α−/− single ligand knockout mice. The systemic anti-IL-1 interventions, IL-1Ra and anti-IL-1α, did not affect neutrophil migration. The IL-1α that drives neutrophil migration is likely derived from CNS cells. IL-1α and platelets mediate neutrophil migration in LPS-induced cerebral inflammation independently of each other.
Chapter 6

General discussion
6.1 Summary of findings

The aims of the experiments in this thesis were to evaluate the role of platelet-derived IL-1α in mediating neutrophil migration \textit{in vivo}. We used four \textit{in vivo} models to investigate the role of platelets and IL-1 in mediating neutrophil migration from the blood into sites of inflammation. Neutrophil migration in peritoneal and air pouch inflammation required the presence of platelets in circulation, yet was independent of IL-1, which eliminated platelet-derived IL-1α as an essential mediator at these sites (Figure 6.1). Neutrophil migration in a model of acute lung injury was independent of both platelets and IL-1 (Figure 6.2).

![Peritoneum & air pouch diagram](image)

\textit{Figure 6.1 – Platelets, but not IL-1, drive neutrophil migration in response to LPS \textit{in vivo} in the peritoneal and air pouch models.}

Platelets are an important mediator of neutrophil migration in the context of LPS-induced peritoneal and air pouch inflammation. IL-1 was not required to mediate neutrophil migration, and the literature implicates macrophage-derived TNFα as an important mediator of vascular inflammation in this context.
Neither platelets nor IL-1 drive neutrophil migration in response to LPS in a model of acute lung injury. Neither IL-1 nor platelets are both important mediators of neutrophil migration in the context of LPS-induced lung inflammation. To migrate into the airway, neutrophils must cross both the vascular endothelium and the specialised airway epithelium. Though contrasting reports exist, the prevailing consensus in the literature implicates TNFα, likely from airway and tissue macrophages, as the key cytokine mediator of neutrophil migration into both the airway and the lung parenchyma. While other chemokines have been implicated, we demonstrated a consistent plasma CXCL1 response. We measured the number of neutrophils in the airway at 6h, and other papers have demonstrated an increase in neutrophil numbers in the lung parenchyma at similar time points.

The brain was the only site where neutrophil migration was dependent on platelets and IL-1. Through use of IL-1α−/− single ligand knockout mice, we demonstrated the requirement of IL-1α in cerebral inflammation. The systemic anti-IL-1 interventions, IL-1Ra and anti-IL-1 antibodies, did not reduce neutrophil migration into the brain parenchyma. This suggested that the IL-1α responsible for neutrophil migration is derived from and/or is acting within the CNS, and ruled out a role for platelet-derived IL-1α in mediating neutrophil migration into the brain. Hence, we are able to propose a refined schematic to that of Thornton et al. (2010) (Figure 6.3).
Figure 6.3 – CNS derived IL-1α and platelets drive neutrophil migration in response to LPS *in vivo*. IL-1α and platelets are both important mediators of neutrophil migration in the context of LPS-induced cerebral inflammation. IL-1α, likely derived centrally from microglia, drives cerebrovascular inflammation at a different stage to platelets, in an alteration to the model proposed by Thornton *et al.* (2010). Further, platelet binding, to endothelium and neutrophils, via GpiIb is a necessary step in the mediation of neutrophil migration.

6.2 Consideration of *in vivo* models

Throughout our experiments, we used LPS as an inflammatory stimulus to induce neutrophil migration, and this choice of model has implications for the results. It was chosen for two reasons. First, it is one of the best characterised inflammatory stimuli in a variety of tissues, including the CNS. Second, in pilot peritonitis experiments *in vivo*, LPS exhibited less variation in inflammatory response than thioglycollate. LPS is a direct agonist of the TLR4 receptors that platelets express (Shashkin *et al.* 2008). There may therefore be variation in the effects of LPS between those models in which LPS reaches the bloodstream and those where it does not. This paper also demonstrated that LPS stimulation directly induces IL-1ß expression in platelets. Use of another
inflammatory stimulus that does not directly activate platelets may have yielded a different result, particularly in the models where LPS reached the circulation.

From a translational perspective, LPS-induced brain inflammation most closely resembles Gram-negative bacterial infection, though doubts have been raised over its suitability to model sepsis (Rittirsch et al. 2007). Relevant acute conditions, during which neutrophils enter the brain, include ischaemic stroke, epilepsy or neuromyelitis optica. LPS is found in none of these, and hence we are not able to make any translational claims with these data; rather, we have tried to uncover a more basic mechanism of neutrophil migration. To further pursue anti-platelet or anti-IL-1 interventions as a possible therapeutic strategy in these conditions the inflammatory stimulus should be altered to be more clinically relevant; for example, injection of DAMPs or dead cells, excitotoxic substances, or experimental autoimmune conditions or stroke.

Our focus on neutrophil migration as an outcome measure has clinical relevance if the influx of these cells into tissues affects disease processes. There is clear evidence for a role of neutrophils in ischaemic brain injury (Chen et al. 1994; Connolly et al. 1996; Dawson et al. 1996; Yenari et al. 1998; Beray-Berthat et al. 2003). Further, a systemic inflammatory stimulus exacerbates ischaemic injury via neutrophil action (McColl et al. 2007). Neutrophil depletion in epilepsy inhibits acute seizures and chronic recurrence, an effect also inhibited by blocking CAMs involved in their migration (Fabene et al. 2008; Sills & Solomon 2009). Given this evidence, our data implicating IL-1α as a key driver of neutrophil migration in the CNS, may have implications for these conditions.

Our initial hypothesis based on previous in vitro findings was that an important mechanism by which platelets facilitate neutrophil migration is via release of IL-1 (Thornton et al. 2010). However, our data show that this is unlikely to be the case in vivo. There is a more complex immunological environment in vivo. The cell culture setup from Thornton et al. (2010) involved only the cells under investigation, the inflammatory stimulus and intervention. In vivo, while the same MBECs were present, there were also peripheral and CNS immune cells, which bring the possibility of many
more inflammatory mechanisms and pathways, which may overwhelm, bypass or inhibit a particular MBEC response to platelet-derived IL-1.

6.3 Tissue-specific mechanisms for driving innate immune responses

We investigated the role of IL-1 in neutrophil migration in four models of LPS-induced inflammation. Neutrophil migration into the brain parenchyma was the only site at which IL-1 was required, indicating tissue-specific requirements for IL-1 in driving innate immune responses and perhaps a unique involvement in the brain.

Differences have been observed in the past between neutrophil recruitment in the brain and other tissues. Recruitment is slower in the brain (Cybulsky et al. 1988; Andersson et al. 1992), which was reflected in our use of a 24h time point in the brain to quantify migrated neutrophils compared to 6h in other tissues. An interesting observation in the literature, that is consistent with our observations of neutrophils in the ventricles after LPS, is that neutrophil recruitment to the ventricular system and choroid plexus occurs independently of recruitment to the brain parenchyma, and at a rate equivalent to recruitment to the peritoneal cavity and skin (Andersson et al. 1992). In addition, we observed IgG in periventricular areas, where it was not present at the injection site, indicating a differing barrier function. A key difference between these brain sites is the composition of the BBB; it has been known for over twenty years that the capillaries in the choroid plexus are fenestrated, and that there are intercellular gaps between endothelial cells (Engelhardt et al. 2001). The composition of the BBB, including the brain-specific tight junctions is a possible reason for the difference in migration rate of neutrophils observed, though we have no data to corroborate this.

Our findings contrast with the consistent requirement for IL-1 across different tissues in sterile inflammation. IL-1 is necessary for neutrophil migration to occur following a sterile inflammatory stimulus in the peritoneum (Chen et al. 2007; Kono et al. 2010), dorsal air pouches (Yang et al. 2002; Jin et al. 2011), the lungs (Kuipers et al. 2012), and following ischaemia in the brain (Allen et al. 2012). There is consistency in the requirement for IL-1 between different anatomical sites where DAMPs are the inflammatory stimulus. PAMPs and DAMPs trigger inflammation through binding to
pattern recognition receptors (PRRs). Different PRRs bind different PAMPs and DAMPs, and initiate different signalling pathways, which accounts for different cellular and cytokine responses to different infections (Takeuchi & Akira 2010). It is likely that PAMP- and DAMP-induced inflammatory processes share a common final effector pathway to mediate neutrophil migration, as it is apparent that the actual process of transmigration is consistent. Where the pathways converge is open to speculation, though it may be at the level of neutrophil mobilisation in response to cytokine action. Another possibility for the difference is that there are multiple inflammatory pathways acting in parallel in peripheral tissues, and that the availability of IL-1 independent pathways meant that genetic knockout of IL-1 had no effect on neutrophil migration. If an IL-1 dependent pathway is the only mechanism for driving neutrophil recruitment in the brain, it may explain our findings. This may be because other pathways are not activated by LPS in the brain, or perhaps that other inflammatory pathways activated do not lead to neutrophil influx as in other tissues. Our data suggest the latter, as in IL-1 knockout mice, neutrophil migration is reduced in response to LPS, while other cytokines remain elevated in response to LPS. This may therefore implicate IL-1α as a key ‘alarmin’ to stimulate the innate immune response in the CNS. There is recognition of this in the literature, where variation in leukocyte recruitment patterns to brain tissue was explained by differential induction of IL-1β and TNFα; IL-1 recruited neutrophils, while TNFα recruited monocytes (Blond et al. 2002). This is not true of recruitment in acute lung injury, where TNFα does mediate neutrophil recruitment (Shimizu et al. 2009). Another difference between the brain and other tissues is its relative sterility; resident immune cells of the lung may be exposed to a wider variety of pathogens more regularly, and therefore have evolved multiple ways to induce neutrophil influx. Further, given the CNS’s limited capacity for repair relative to other tissues, it may have different mechanisms for ensuring clearance of infection. It is clear that, overall, the importance of different mechanisms governing neutrophil recruitment is highly context dependent, and varies by inflammatory site and stimulus.

The identification of IL-1α as a potential target to prevent neutrophil migration in the CNS, raises several questions about how best to develop a therapeutic strategy. Drug delivery specifically into the CNS is a challenge, particularly when, as with IL-1α, there
may be implications of altering systemic immune function. While it is possible to administer compounds directly to the CNS, these would likely need extensive clinical support, and thus may not be feasible. In addition, few anti-inflammatory interventions are truly tissue-specific (Lipworth 1996; Weiss 2011), though the possibility of a tissue-specific mediator may allow more specific targeting. Our data also suggest that targeting CNS-derived IL-1 may not impair the inflammatory response to peripheral challenges.

6.4 Platelets as therapeutic targets in vascular inflammation

Platelets were consistently required for neutrophil migration in all three of the models we used, with the exception of the lungs. We are also the first to show a requirement for platelets in mediating neutrophil migration into the brain parenchyma in vivo. This raises the possibility that there is a consistent platelet mechanism for driving neutrophil migration in different vascular beds, which may be of great significance in the wide range of conditions that involve inflammation of the blood vessel wall. We found that anti-GpIb antibody reduced neutrophil migration in the brain, which implicates platelet interaction with neutrophils and endothelial cells as an important factor, a finding in common with a paper showing a role for platelets in experimental autoimmune encephalitis (Langer et al. 2012). In the brain, there are reports that blocking adhesion molecules including selectins and PECAM-1 reduces leukocyte recruitment (Connolly et al. 1997; Bernardes-Silva et al. 2001; Tiwari et al. 2009). It is unlikely that any one particular adhesion molecule will be isolated as responsible, and many molecules are likely to facilitate cellular interactions in positive feedback.

It is notable that we repeatedly observed that the depletion of platelets also abrogated increase in plasma IL-1α induced by LPS. This is consistent with recent papers demonstrating that platelets are a source of IL-1α (Boilard et al. 2010; Thornton et al. 2010). Given the degree of IL-1α reduction, we may venture to suggest they may be the major source of IL-1α following LPS-induced inflammation; though the possibility remains they may trigger its release rather than producing it themselves.
6.5 Future directions

There remain questions to answer regarding the role of IL-1 and platelets in mediating neutrophil migration in cerebral inflammation. If CNS-derived IL-1α mediates neutrophil migration, we have yet to definitively demonstrate the source of the cytokine and its cellular target during this process. The use of cell-specific knockdown mice may be appropriate for this purpose. Cre-lox transgenic technology has been used to generate cell-specific knockout mice, in a variety of brain cell types, including microglia (Ros-Bernal et al. 2011; Mobley & McCarty 2012). Mice lacking IL-1α in microglia could be generated, in which to test our hypothesis that these cells generate the CNS-derived IL-1α that mediates neutrophil migration. As microglia are derived from a myeloid lineage (Ginhoux et al. 2010), cre-lox mice may need to be combined with the generation of bone marrow chimaeras to specifically isolate microglia-derived IL-1α. However, this would be a complex experiment and given pre-existing data, using IL-1α bone marrow chimaeras alone may provide strong evidence to at least firmly establish if CNS or non-CNS sources are critical. Thornton et al. (2010) showed that the MBEC response to IL-1α facilitates migration in vitro. Endothelial cell specific knockdown of IL-1α’s receptor, IL-1R1, has been used previously to characterise inflammatory responses (Ching et al. 2007), and could be used to identify if these cells are the target for IL-1α in LPS-induced cerebral inflammation.

The identification of IL-1α as a key mediator of neutrophil migration in cerebral inflammation may have implications for medical conditions where neutrophil migration contributes to CNS pathology, such as cerebral ischaemia (McColl et al. 2007; Allen et al. 2012). Previous translational studies have shown ischaemic damage in IL-1α single ligand knockout mice is not reduced compared to wild-type mice (Boutin et al. 2001); neutrophil migration into brain tissue was not quantified. Interventions targeting IL-1 actions are in pre-clinical and clinical development for treating a variety of inflammatory conditions, but primarily focus on blocking the IL-1β ligand. Future work may highlight the equally important pathological role of IL-1α and therefore indicate that interventions blocking actions of both IL-1 ligands (e.g. receptor antagonism) are needed. Although removal of IL-1α may not be sufficient to reduce ischaemic damage,
future translational experiments may identify a role for targeting IL-1α as part of a combinatorial therapeutic strategy, for which a systems biology approach to translational research may prove fruitful.

We demonstrated the possibility of tissue-specific mechanisms for driving innate immune responses. A high-throughput approach, using arrays for a comparative analysis of inflammatory profiles in different tissues may further elucidate differences and their underlying mechanisms. Another approach may be to culture the resident immune cells of different tissues and subject them to inflammatory stimuli. Based on our data, we may hypothesise that microglia might exhibit a different IL-1α response to the equivalent cells of other tissues.

In three of the models we used, platelets in circulation were needed for neutrophil migration. Our data show that the mechanism by which platelets mediate migration is not via IL-1; however, there may be a common platelet mechanism that facilitates the process, providing another question for future exploration. Platelets can influence leukocyte recruitment to inflamed tissue through adhesive interactions with endothelial cells and leukocytes (Gawaz et al. 2005). A more detailed evaluation could be undertaken into platelet-leukocyte-endothelial interactions in the cerebrovasculature in response to LPS injection or other inflammatory stimuli, using experimental strategies to isolate different aspects of platelet function that have been implicated in neutrophil migration, such as interactions mediated by PSGL-1. In addition to immunohistochemistry, multi-photon imaging could be used to visualise dynamic cellular interactions in situ (Svoboda & Yasuda 2006). This technique has been used before to investigate platelet-leukocyte interactions in the brain, and neutrophil migration in other organs (McDonald et al. 2010; Jenne et al. 2011). This would enable more detailed analysis of the stages of neutrophil migration, including rolling, firm adhesion and transmigration.

In summary, our data implicate platelets and IL-1α as important mediators of different stages of the recruitment of neutrophils to the CNS in response to LPS. This may have implications for the development of therapeutics in neurological conditions where
neutrophils mediate brain injury. The data raise further questions about the source and target cells of IL-1α in the CNS, a general role for platelets in mediating neutrophil migration throughout the body, and the intriguing possibility of tissue-specific mechanisms for driving innate immune responses.
Chapter 7

Appendices
7.1 Solutions

0.4M phosphate buffer
57.29g disodium hydrogen orthophosphate dihydrate (Na$_2$HPO$_4$·2H$_2$O) and 11.03g sodium dihydrogen orthophosphate hydrate (NaH$_2$PO$_4$·H$_2$O) per litre dH$_2$O.

4% paraformaldehyde (PFA)
3 granules NaOH in 500ml dH$_2$O at 60ºC. Add 40g paraformaldehyde powder. Once dissolved, add 250ml 0.4M PB and 250ml dH$_2$O. Sterile filter and adjust to pH 7.4.

Reagent diluent for immunohistochemistry
600µl Triton in 40ml PBS at 50ºC. Add 160ml PBS, 20µg NaN$_3$ (Sigma, UK).

0.2M sodium acetate
1.641g anhydrous sodium acetate (CH$_3$COONa) (Fischer, UK) per 100ml dH$_2$O. Adjust to pH 6.0 with glacial acetic acid (Fischer, UK).

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

Cryoprotectant
6.6g disodium hydrogen orthophosphate dihydrate (Na$_2$HPO$_4$·2H$_2$O) and 0.79g sodium dihydrogen orthophosphate hydrate (NaH$_2$PO$_4$·H$_2$O) in 500ml dH$_2$O. Add 300ml anhydrous ethylene glycol (Sigma, UK) and 200ml glycerol (Fischer, UK).

Homogenisation buffer
7.88g NH$_4$Cl (Fischer, UK), 1g KHCO$_3$ (Fischer, UK) and 0.367 Na$_2$-EDTA (Fischer, UK) per litre dH$_2$O. Adjust pH to 7.4 with 1M HCl.
7.2 Flow cytometry gating strategy for neutrophils

Representative gating strategy for Ly-6G-positive neutrophils (Figure 7.1). Gating by pulse width removed aggregated cells. Gating by low FSC, high SSC removed fragments. Gating for Ly-6G positive staining was used to quantify neutrophils.

7.3 Power calculations

Power calculations to determine group size, based on pilot experiments for treatment effect and variation, were performed as follows:

\[ n = \left( \sum \alpha + \sum \beta \right)^2 \cdot \left( \frac{2\sigma^2}{(\bar{x}_1 - \bar{x}_2)^2} \right) \]

where \( \alpha=0.025 \) (two-tailed test; \( \sum\alpha=1.96 \)), \( \beta=0.2 \) (\( \sum\beta=0.8416 \))
7.4 Effect of anti-CD41 antibody on circulating monocytes

We raised concern in Chapter 3 that anti-CD41 antibody decreased the number of monocytes in circulation. While the effect did not reach statistical significance, a reduction in monocytes could be a confounding factor. We repeated the characterisation of anti-CD41 antibody, injecting C57BL/6 mice with anti-CD41 antibody (n=3) or IgG isotype control (n=3). Injection of anti-CD41 antibody had no significant effect on the total population of monocytes (Gr-1+/SSlo), and in this repeat, there was a non-significant increase in the number of circulating monocytes (Figure 7.2).

![Figure 7.2 – Effect of anti-CD41 antibody on circulating monocytes.](image)

Mice were injected with anti-CD41 antibody or IgG control. At 24h mice were culled, and the number of monocytes in a cardiac blood sample was quantified by flow cytometry for Gr-1+ cells with a low side scatter.
7.5 Pilot experiment: LPS-induced cerebral inflammation

A pilot experiment was conducted first to determine the effect size and variation for power analyses of LPS in stimulating neutrophil migration into brain tissue, we injected the striatum of two groups of C57BL/6J mice with vehicle (n=3) or LPS (1µl of 4mg/ml, n=3) to induce inflammation. Figure 7.3 shows representative DAB immunohistochemistry for neutrophils (SJC4-positive). Neutrophils migrated into the ipsilateral brain parenchyma in response to LPS, with a florid infiltration of the meninges and choroid plexus.

**Figure 7.3 – Neutrophils in the brain parenchyma in response to LPS.**
Representative DAB immunohistochemistry for neutrophils (SJC4-positive). (a,c) PBS injected mice. (b,d) LPS injected mice.
7.6 BBB permeability in response to striatal LPS injection

To determine whether LPS injection induced BBB breakdown, we stained brain slices from the cerebral anti-IL-1 antibody experiment for mouse IgG. The majority of sections exhibited periventricular IgG staining, in concordance with the distribution observed in a previous study (Andersson et al. 1992). There were no differences between the groups. A semi-quantitative analysis was undertaken, whereby degree of staining was classified into three groups: no staining, periventricular staining, and major staining. There distribution of IgG between treatment groups, and between PBS and LPS injected mice was similar (Figure 7.4).

Figure 7.4 – Analysis of BBB breakdown, via mouse IgG staining, in response to cerebral LPS injection.

Mice were pre-treated with anti-IL-1 antibodies or IgG control 24h before striatal injection of LPS or vehicle. Brains were removed at 24h and stained for mouse IgG to indicate BBB breakdown. Degree of IgG staining was classified into three groups. Classification by treatment group and injection are shown, and representative staining for each classification.
7.7 IL-1Ra in the brain parenchyma following antibody treatment

To determine whether recombinant human IL-1Ra injected systemically reached the brain parenchyma, we stained brain tissue with anti-huIL-1Ra antibody. No IL-1Ra was detected in the striatal injection site or other brain areas at 24h. At the point of microneedle entry on the surface of the cortex, IL-1Ra was detected in some slices, which reflects probable leak from the systemic circulation due to bleeding (Figure 7.5).

![Figure 7.5 – Analysis of IL-1Ra in brain tissue following i.p. injection.](image)

Mice were injected i.p. with recombinant human IL-1Ra before and after LPS striatal injection. Brains were removed at 24h and stained for huIL-1Ra. (a) Representative staining of the striatum all animals receiving IL-1Ra. (b) In some slices, IL-1Ra was detected at the surface of the brain at the point of microneedle entry.
7.8 No rat IgG in the brain parenchyma following antibody treatment

To determine whether anti-IL-1 antibodies penetrated the brain parenchyma at 24h, we stained brain slices from the cerebral anti-IL-1 antibody experiment for rat IgG. No rat IgG was detected in brain slices from any group (Figure 7.6).

Figure 7.6 – Lack of penetrance of anti-IL-1 antibodies into brain tissue.
Mice were pre-treated with anti-IL-1 antibodies or IgG control 24h before striatal injection of LPS or vehicle. Brains were removed at 24h and stained for rat IgG to indicate penetrance of the brain parenchyma by the antibodies. Representative staining of all groups is shown above via seven serial brain slices around the coronal level of the injection, with no detectable rat IgG.
Chapter 8

References


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