Regulation of Inflammation by the Fps/Fes Protein Tyrosine Kinase

By

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Abstract

Fps/Fes and Fer are members of a distinct subfamily of cytoplasmic protein tyrosine kinases that have recently been implicated in the regulation of innate immunity. Previous studies showed that mice lacking Fps/Fes are hyper-sensitive to systemic lipopolysaccharide (LPS) challenge. This study identifies physiological, cellular and molecular defects that contribute to the hyper-inflammatory phenotype in Fps/Fes-null mice. We showed that plasma tumour necrosis factor (TNF)-α levels were elevated in LPS challenged Fps/Fes-null mice as compared to wild type mice, and cultured Fps/Fes-null peritoneal macrophages treated with LPS showed increased TNF-α production. Cultured Fps/Fes-null macrophages also displayed prolonged LPS-induced degradation of IκB-α, increased phosphorylation of the p65 subunit of NF-κB, and defective TLR4 internalization. Next, we showed a role for Fps in the regulation of recruitment of inflammatory leukocytes. Using the cremaster muscle intravital microscopy model, we observed increased leukocyte adherence to venules, and increased rates and degrees of transendothelial migration in Fps/Fes-null mice, compared to wild type. There was also increased neutrophil migration into the peritoneal cavity subsequent to thioglycollate challenge. Using flow cytometry, we observed prolonged expression of the selectin ligand PSGL-1 on peripheral blood neutrophils from Fps/Fes-knockout mice stimulated ex-vivo with LPS. Finally, we examined the role of Fps/Fes in regulating apoptosis in response to inflammation. Upon intra-peritoneal challenge with LPS, Fps/Fes-null mice displayed a decreased depletion of macrophages from the peritoneal cavity. In response to ex-vivo TNF-α stimulation, macrophages from Fps/Fes-null mice underwent decreased apoptosis and necrosis as assessed by flow cytometry. Immunoblot analysis revealed that Fps/Fes-null macrophages displayed more TNF-α-induced degradation of IκB-α in Fps/Fes-null cells, with corresponding increases in the phosphorylation of the p65 subunit of NF-κB. In addition, stimulation of macrophages with TNF-α up-regulated PARP expression in wild-type macrophages; this up-regulation was not observed in Fps/Fes-null macrophages. Finally we
observed a decreased recruitment of macrophages to the peritoneal cavities of Fps/Fes-null mice, with a corresponding increase in neutrophil recruitment, 5 days after thioglycollate challenge. Overall, we show that there is a role for Fps/Fes in regulating inflammation at the physiological, cellular, and molecular levels, and that this might be relevant in inflammatory disease.
Co-Authorship

The co-authors of this thesis are Jeffrey Mewburn, Peter Truesdell, Nadia Griller, Sean Devine, and Peter Greer. The contributions that these authors made to the experimental work, writing, and editing of the manuscripts are listed below.

Chapter 2: This paper was published in the Journal of Leukocyte Biology, in December 2006. The authors are Sean Parsons and Peter Greer. I performed all of the experiments for the manuscript. I wrote the initial draft of the manuscript, and participated in some of the editing. The bulk of the editing of this manuscript was done by Peter Greer.

Chapter 3: This paper has been accepted for publication, and is currently in press in Immunology. The authors of this paper are Sean Parsons, Jeffrey Mewburn, Peter Truesdell, and Peter Greer. Jeffrey Mewburn collected the intra-vital microscopy data, and wrote the materials and methods section pertaining to this technique. He and I analysed the intra-vital microscopy data together. Peter Truesdell contributed to the acquisition of data for Figure 3-4. All other experiments were performed by me. I wrote the initial draft of the manuscript, and participated in subsequent editing. The bulk of the editing of this manuscript was done by Peter Greer.

Chapter 4: This manuscript has not yet been sent out for peer review. The authors of this paper are Sean Parsons, Nadia Griller, Sean Devine, and Peter Greer. Sean Devine assisted me in acquiring the data for Figure 4-2. Nadia Griller acquired most of the data for Figure 4-4, and participated in writing the results section pertaining to this data. All other experiments were done by me. I wrote the initial draft of the manuscript, and participated in subsequent editing. The bulk of the editing was done by Peter Greer.
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<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variation</td>
</tr>
<tr>
<td>CD</td>
<td>Cellular differentiation antigen (eg. CD11b)</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CIP4</td>
<td>CDC42 interacting protein 4</td>
</tr>
<tr>
<td>CME</td>
<td>Clathrin mediated endocytosis</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E.coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EFC</td>
<td>Extended FCH domain</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FCH</td>
<td>Fps/Fer/CIP4 homology domain</td>
</tr>
<tr>
<td>Fer</td>
<td>Fes related</td>
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<tr>
<td>Fes</td>
<td>Feline sarcoma</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fps</td>
<td>Fujinami poultry sarcoma</td>
</tr>
<tr>
<td>FSV</td>
<td>Fujinami sarcoma virus</td>
</tr>
<tr>
<td>GAG</td>
<td>group associated antigen</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HS-1</td>
<td>Hematopoietic lineage specific protein 1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon (eg. interferon-γ)</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of κB</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin (eg. interleukin-10)</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MD2</td>
<td>Myeloid differentiation antigen 2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBN</td>
<td>Peripheral blood neutrophil</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand 1</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RLOT</td>
<td>Reflected light oblique transillumination</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll like receptor 4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>TNFR1</td>
<td>TNF receptor 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Chapter 1

General Introduction

1.0 Objectives

Inflammation is a process that is initiated by the body in response to tissue injury and infection. Inflammation is critical in order for the host to resolve infection and repair tissue. A heightened sensitivity of Fps-null mice to endotoxin challenge first suggested a role for the proto-oncogene $fps$ in the regulation of inflammation. The objective of my research was to elucidate how the Fps protein tyrosine kinase contributes to the regulation of inflammation at the systemic, cellular, and molecular levels.

1.1 The $fps/fes$ gene

1.1.1 Discovery of the $fps/fes$ proto-oncogene

The discovery of the $fps$ and $fes$ genes originated with the isolation of retroviral oncogenes from avian[1] and feline[2] sarcomas, respectively. Subsequent sequence analysis of the cellular counterparts of these oncogenes revealed that they corresponded to orthologues of the same proto-oncogene[3-6]. For simplicity, I will refer to this gene as $fps$ and the protein as Fps for the remainder of this thesis.

Viral $fps/fes$ oncogenes code for chimeric proteins consisting of retroviral group associated antigen (GAG) sequences fused to varying portions of the cellular Fps protein (Figure 1-1). The addition of GAG sequences to Fps results in unregulated tyrosine kinase activity, which is thought to come about through localization of Fps to the plasma membrane[7], or through interference with a regulatory function of the N-terminal domain[8, 9].
1.1.2 Predicted Structure of the Fps protein

The cellular Fps protein tyrosine kinase consists of an amino-terminal Fer/Fps/CIP4 homology (FCH) domain, followed by two predicted coiled-coil domains, a central Src homology 2 (SH2) domain, and a carboxy-terminal kinase domain (Figure 1-1). The FCH and two predicted coiled-coil domains collectively make up an extended FCH (EFC) domain.

1.1.2.1 FCH and coiled-coil domains or EFC/F-BAR domain

The FCH domain at the N-terminus of the Fps protein was first described as a region of homology between the closely related Fps and Fer protein tyrosine kinases (PTKs) and a CDC42-interacting protein 4 (CIP4)[10]. This domain is now recognized in many proteins, where its function has been attributed to cytoskeletal rearrangements, vesicular transport, and endocytosis[11-14]. The FCH domain of Fps has also been shown to be essential for association of Fps with microtubules[15, 16].

The FCH domain of Fps is followed by a predicted region of two coiled-coil domains[17], and this domain is has been suggested to regulate the formation of pentamer or higher-order oligomers of the Fps protein[17]. Oligomerization of Fps promotes autophosphorylation of the protein[17], and this is thought to be regulated through trans-interactions of the coiled-coil domains of adjacent Fps proteins[17]. Intramolecular coiled-coil interactions are also thought to be important in the negative regulation of Fps function, since disruption of the first coiled-coil domain resulted in increased Fps kinase, transforming, and differentiation-inducing activities[8, 18]. An overall model has been proposed whereby cis-interaction (as well as heterotypic trans-interaction) of coiled-coil domains restrains Fps kinase activity, and disruption of this cis-interaction, or trans-interaction with a heterologous EFC domain-containing protein, allows for homotypic (between two or more Fps molecules) trans-interaction of coiled-coil domains, resulting in autophosphorylation and increased Fps activity[8].
In addition to regulation of kinase activity, the coiled-coil domains of Fps have been suggested to be necessary for the differentiation-induced nuclear localization of this protein[19]; however, this putative role of the coiled-coil domain may be specific to activated cells of the myeloid lineage [19, 20].

More recent investigations have revealed that the FCH domain of many proteins is followed by a series of coiled-coils, which collectively make up a novel protein domain called an FCH-BAR (F-BAR) [21] or extended FCH (EFC) domain, that bind a variety of phospholipids with high affinity[22]. The recently solved crystal structure of CIP4 and FBP17 EFC domains predicts a dimer, making up a curved helical bundle with a positively charged concave surface capable of interacting with phospholipid-containing membranes[23], and also implicated in the regulation of actin dynamics and membrane invagination[21]. These EFC dimers were also predicted to form a long curved filament, through end-to-end interaction, and these curved filaments might then stack upon one-another to form a tube-like structure [23]. Therefore, the regulation of Fps activity by its coiled-coil domains is probably the result of EFC-EFC interactions. These interactions could possibly negatively regulate Fps activity, either by cis-interactions (the Fps EFC domain with itself) or heterotypic trans-interactions (the Fps EFC domain with the EFC domain of another protein); alternatively, these EFC-EFC interactions could positively regulate Fps activity, through homotypic trans-interactions (Fps EFC domain with another Fps EFC domain), thereby allowing trans-phosphorylation between the kinase domains of two or more Fps molecules (Figure 1-2).

1.1.2.2 SH2 domain

The SH2 domain is a highly conserved protein module, and is part of a larger family of protein modules that are involved in modulating protein-protein interactions within the cell[24]. The SH2 domain was originally described as a non-catalytic domain in Fujinami Sarcoma virus
(FSV) GAG-Fps about 100 amino acids in length, that could modulate its transforming ability and kinase activity, and was necessary for substrate recognition[25-27]. It has since been shown that the SH2 domains of Fps (and other proteins), bind to phosphotyrosine moieties with high affinity, and that specificity for this interaction is provided by adjacent residues[24, 28]. Furthermore, SH2 domains are located directly N-terminal to the kinase domains in the majority of cytoplasmic protein tyrosine kinases[29].

Other studies specific to the Fps protein itself have provided insights into the function of its SH2 domain in regulation and function of the protein. In FSV GAG-Fps, the SH2 domain has been shown to interact with the kinase domain, whereby it promotes kinase function, and is also required for association with cellular substrates[30]. Studies with human Fps have shown that this SH2-kinase domain interaction is dependant upon autophosphorylation of the protein[31]. Phosphopeptide library screening has identified pYEXV/I (single letter amino acid nomenclature, in which pY represents phosphorylated tyrosine, and X represents any amino acid) as the optimal consensus sequence for binding of the Fps SH2 domain[32]. It is interesting to note that although this analysis identified sequences in many proteins as potential binding sites for the Fps SH2 domain (including sites in focal adhesion kinase (FAK), Tec, Lyn, Abelson murine leukemia viral oncogene homologue (Abl), hematopoietic cell kinase (Hck), CD72, CD3ε, SH2 domain containing protein tyrosine phosphatase 1 (SHPTP-1), leukocyte antigen related protein tyrosine phosphatase (LAR-PTP), ezrin, breakpoint cluster region (Bcr)[33], 3BP2A and γ-adaptin), there is little experimental evidence to suggest that these interactions take place in vivo. However, there is also a consensus binding site for the Fps SH2 domain within the α-chain of the mouse FcγRI receptor[32]. This receptor shares a common FcR γ-chain with the FcεRI receptor on mast cells and the GPIV receptor on platelets. Interestingly, Fps and Fer have been shown to be involved in signalling downstream of both the FcεRI receptor in mast cells[34], and the GPIV receptor in platelets[35].
Figure 1-1 Structure of cellular Fps and Fer proteins, and several viral Fps oncogenes. Fps and Fer share a similar structural organization, with an N-terminal Fps/Fer/CIP4 (FCH) domain (yellow oval), followed by 2 regions of predicted coiled-coil domains (green hexagon), a more central Src homology 2 (SH2) domain (blue pentagon), and a C-terminal kinase domain (red rectangle). As noted, the FCH and coiled-coil domains together make up the extended FCH or EFC domain. Also shown are the domain structures of three of the transforming viral Fps homologues, including the feline Gardner-Arnstein (GA) GAG-Fes, the chicken PRClI and Fujinami sarcoma virus (FSV), all of which include an N-terminal Gag sequence (purple rectangle). [Adapted from P.A. Greer, Nat. Rev. Mol. Cell. Biol., 2002[36]]
Figure 1-2  Regulation of Fps kinase activity by EFC domains. Represented are two inactive configurations of Fps; one in which there are cis-interactions within the EFC domain, and a second in which there are trans-interactions between the EFC domains of Fps and an as of yet unidentified protein X. Both of these proposed configurations prevent the formation of an oligomeric Fps structure, which is thought to activate the protein by allowing for trans-phosphorylation between adjacent Fps molecules[37]. The EFC domain of Fps might be responsible for regulating these homotypic intermolecular interactions.
1.1.2.3 Kinase domain

Using a degenerate peptide library, Songyang et al. [32] mapped the in vitro peptide substrates of nine separate protein tyrosine kinases, including Fps. Interestingly, they found that these protein tyrosine kinases preferentially phosphorylated peptides that were recognized by their own SH2 domains[38]. Therefore, all of the potential binding partners listed above for the Fps SH2 domain also represent potential substrates for its kinase activity.

Within its kinase domain, Fps can be phosphorylated on tyrosine 713, and tyrosine 811. Mutation of tyrosine 713 led to a decrease in the autophosphorylation of 811, as well as a decrease in the transphosphorylation of the Fps substrate BCR, thus revealing tyrosine 713 as important for the regulation of Fps kinase activity[39]. Phosphorylation at this site has been proposed to occur via both an intramolecular interaction involving the SH2 domain[31] and also via an intermolecular interaction[39]. The question of whether or not Fps autophosphorylation involves intramolecular SH2-kinase domain interactions has not yet been conclusively answered. However, one recent study has elucidated a 3 dimensional structure for the Fps SH2 domain[40], while another has succeeded in crystallizing the kinase domain[41]. Therefore, forthcoming three dimensional models of the Fps kinase and SH2 domains might further elucidate the structural basis of Fps autophosphorylation at tyrosine 713.

1.1.3 Expression pattern and sub-cellular localization of Fps

Cellular Fps expression was first detected in myeloid cells, where its expression was highest in cells of the monocyte-macrophage lineage[42, 43]. Its expression has also been described in neutrophils[44], mast cells[45], platelets[35], erythrocytes[46], vascular endothelial cells[47], neuronal cells, and several epithelial cell types, including those of the choroid plexus and the uterus[48]. As well, during development Fps is expressed in all three germ layers[48].
More recently, our lab has found evidence for abundant expression of Fps in lactating mammary tissue, where it is also highly activated (Truesdell and Greer, unpublished observations).

Both cellular and viral Fps were originally described as being localized to the cytoplasm[49-51]. Fps localization has also been described at the trans-golgi network[20, 48], or other areas involved in vesicular trafficking[20]. In contrast, other reports have suggested that it may be expressed in the nucleus[52], but that nuclear expression might be specific activated cells of the myeloid lineage [19]. Most of the studies that concluded Fps localization is restricted to the cytoplasm examined the localization of cellular or viral Fps introduced into fibroblasts or myeloblasts[20, 49-51]. On the other hand, a later study concluded that Fps nuclear localization occurred in myeloid cells, but not in fibroblasts[19]. This might suggest that these two findings need not be mutually exclusive; however, a study by Haigh et al. detected Fps localization to the perinuclear, but not intranuclear region in cells of the myeloid lineage[48].

1.1.4 Cellular functions of the Fps protein

1.1.4.1 Cellular transformation and cytokine signalling

Most of the early structure-function work on the Fps protein employed the use of the FSV-encoded GAG-Fps oncoprotein. One of the first findings to come out of this early work was that the ability of FSV GAG-Fps to induce transformation in cells was dependent on its ability to phosphorylate tyrosine residues on substrate proteins[53], and to undergo tyrosine phosphorylation itself[54]. Later studies showed that infection with viral GAG-Fps could induce both myeloid differentiation[55] and growth[56], without the need for exogenous growth factors, thereby suggesting that Fps might function in growth factor signalling. Indeed, later work produced evidence of the induction of kinase activity and tyrosine phosphorylation of cellular Fps in cells stimulated with interleukin 3 (IL-3) and granulocyte/macrophage colony stimulating
factor (GM-CSF)[57], two cytokines involved in myeloid cell maturation. Soon after, this same group showed that stimulation with erythropoietin, which is involved in maturation and proliferation of erythroid cells, could also induce Fps activation[58]. This suggested that Fps might play a more general role in the transmission of signals for cellular growth and differentiation. Since this time, there have been a number of reports suggesting activation of Fps after stimulation with GM-CSF[44], IL-4[59], IL-6[60], stem cell factor[46], fibroblast growth factor 2 (FGF2)[61], vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF)[62, 63], and semaphorin3A[61].

1.1.4.2 Myeloid survival and differentiation

The high expression of Fps in myeloid cells and their progenitors suggested that Fps might play a role in myeloid cell differentiation, and possibly survival as well. Indeed, some of the studies mentioned above seemed to indicate this, since the viral Fps could induce differentiation in several cell lines[55, 56]. Later studies showed that inhibition of the cellular Fps protein resulted in a retardation of granulocytic differentiation due to increased apoptotic cell death[64], and that introduction of cellular Fps into a leukemic cell line allowed for myeloid differentiation[65]. These studies seemed to support a role for Fps in the differentiation of myeloid cells. However, more recent studies using gene-targeted fps knockout or knockin mice have shown that Fps is not essential for myeloid differentiation, and loss of either Fps kinase activity or Fps expression resulted in only subtle effects on the mouse hematopoietic differentiation program[66, 67]. These mouse models will be discussed in further detail below.
1.1.4.3 Receptor down-regulation

Anderson and Ismail examined the role of viral Fps in transformation through activation of the PDGF receptor[68]. Interestingly, their observations suggested a role for viral Fps in down regulation of the receptor. An approximate 4-8 fold down regulation was observed at the mRNA level using an RNase protection assay; however a greater than 100 fold decrease at the protein level was observed by western blot analysis, suggesting that transcriptional regulation alone is not responsible for the complete down regulation of the receptor protein. In both cases, the kinase activity of the viral Fps protein was required for down regulation. Therefore, one of the suggestions the authors put forward for how viral Fps might be inducing down regulation, is through the constant phosphorylation, and therefore internalization/degradation of the receptor molecules[68].

We have since proposed a more general role for Fps in the down regulation of receptors; briefly, we found that macrophages isolated from Fps-null mice, displayed a reduced ability to down-regulate surface receptors (both toll like receptor 4 (TLR4) and transferrin receptor) subsequent to stimulation. In chapter 2, we suggest that this might be due to a possible interaction of Fps with cytoskeleton-regulating proteins, such as HS-1 and or dynamin. For a more complete discussion, see section 2.4.

1.1.4.4 Regulation of the cytoskeleton

Possibly one of the first indications that Fps might participate in cytoskeletal regulation, came from work done by Young and Martin, which indicated that the transforming ability of proteins containing Fps sequences correlated with their association with cellular structural components[69]. This idea of how Fps might participate in cytoskeletal regulation has since been refined[36]. In the context of myeloid cells, Fps may participate in the phosphorylation/activation of hematopoietic lineage specific protein 1 (HS-1), a cortactin homolog that participates in the
regulation of cortical actin dynamics. A more in-depth discussion on this topic is provided in chapter 2.

1.1.5 Mouse genetic models examining Fps function

One of the first uses of transgenic mouse techniques to examine Fps function was by Yee et al. in 1989, in which an FSV GAG-Fps transgene was introduced into the mouse germline[70]. This approach resulted in mice developing a wide array of tumours, including lymphomas, thymomas, fibrosarcomas, hemangiomas, and angiosarcomas. However, some of the tissues that expressed the viral Fps oncogene failed to develop tumours, suggesting that other genetic events are required for oncogenesis, in addition to the mutation of the Fps kinase. Since then, several other genetic mouse models have been produced to examine the role of the fps gene.

1.1.5.1 Myristoylated Fps (fpsMF)

The next mouse model used to examine the function of Fps, was one in which a human genomic DNA fragment containing all the fps coding sequence was used to generate transgenic mice by zygote microinjection[71]. Tissue-specific over-expression of Fps was observed in these mice; which confirmed that the transgene contained the complete promoter, including all transcriptional regulatory sequences. However, these mice had no overt phenotype. So a new transgenic line was created using this same human DNA fragment, in which additional coding sequences were added to provide an N-terminal myristoylation sequence[47]. It was hoped that this fatty acylation modification would subtly activate Fps, leading to some phenotypes in the mice that would help elucidate its normal physiological function [47].

These mice displayed hypervascularity which progressed into vascular tumours[47], possibly due to increased sensitivity to VEGF and PDGF[62]. Increases in signalling downstream
of GM-CSF with associated increases in the numbers of circulating granulocytic and monocytic cells[72], and increased erythropoietin and stem cell factor sensitivity, with increased numbers of immature erythroid progenitors[46], were also observed in these mice. These observations argued strongly for a role for Fps in the regulation of both hematopoiesis, and angiogenesis.

1.1.5.2 Kinase-inactive knockin mutant (fps\textsuperscript{K>R/K>R})

In this model, a missense mutation was introduced into the fps gene by gene targeting, resulting in a lysine to arginine (K588>R) knockin mutation within the kinase domain. This lysine is essential for kinase function, and the mutation resulted in a kinase-inactive mutant Fps protein in transgenic mice. The mutant Fps protein was expressed at the same levels as the wild type protein in mice[66]. Mice homozygous for the fps\textsuperscript{K>R} allele displayed no overt phenotypes; however, in bone marrow hematopoietic progenitor colony forming assays, the lack of Fps kinase activity resulted in slightly elevated numbers of colony forming units-granulocyte macrophage in response to IL-3 and GM-CSF. Furthermore, there was a decrease in signalling downstream of GM-CSF in macrophages derived from the bone marrow of these mice. This model again suggested a role for Fps in hematopoiesis, and in signalling downstream of some of the cytokines that regulate this process.

1.1.5.3 Fps-null

To date, two mouse models have been reported in which the fps gene has been targeted so that no stable Fps protein is produced. The first, reported by Hackenmiller et al., resulted in homozygous mice that were viable, but generally unhealthy and not born in the expected Mendelian ratios[73]. These mice also displayed increases in the number of monocytes and neutrophils, and decreases in the number of B lymphocytes. Macrophages from these mice also
showed increased adhesiveness, and increased responsiveness to stimulation with GM-CSF and IL-6[73]. These results seemed to suggest a role for Fps in regulating hematopoiesis, and in signalling downstream of some of the cytokines which regulate this process.

The second mouse model to incorporate a null mutation into the fps gene, was one reported by Zirngibl et al[67]. In this model, a mutation was introduced that resulted in deletion of sequences encoding the kinase domain in the 3’ end of the gene. This resulted in the elimination of Fps protein expression, thereby producing a Fps-null mutation (for the remainder of this thesis, this model will be referred to as Fps-null when referring to the protein or the transgenic mouse, and fps−/− when referring to the gene and for labelling figures). Interestingly, in contrast to the previous report by Hackenmiller and colleagues, these researchers found that Fps-null mice were born in the expected Mendelian ratios, and were healthy and fertile. Almost completely opposite to the Hackenmiller model, these mice showed a modest decrease in the levels of granulocytes and monocytes, and only a very slight decrease, or in some cases an increase, in the levels of B lymphocytes, depending on the physiological compartment analyzed. As noted by the authors, the differences in these observations might be due to a faulty targeting strategy in the Hackenmiller model, in which the closely linked furin gene, known to participate in embryogenesis, could have been modified due to the proximity of its 3’ end to the fps gene’s transcription start site.

Again in contrast to the Hackenmiller knockout model, the second Fps-null model displayed no overt defects in signalling downstream of stimulation with GM-CSF, IL-3 or IL-6[67]. However, although both groups performed their experiments in macrophages cultured from bone marrow, the culture conditions between the two groups were different. Specifically, there appears to be differences in the levels of fetal bovine serum (FBS), GM-CSF and IL-3 used to induce maturation into macrophages, as well as differences in the culture times and passaging strategies. Therefore, the discrepancies observed between these two groups with respect to Fps’
role in cytokine signalling in macrophages, could be due to the two groups using slightly different cell populations. Both of the above models are suggestive of a role for Fps in the regulation of hematopoiesis, although the magnitude and direction in which it does so remains in question.

One aspect of agreement between the two Fps knockout models was that both supported a role for Fps in regulating innate immunity. Hackenmiller et al. reported that Fps knockout animals had an increase in ankle swelling in response to *B. burgdorferi* infection[73], while the second report showed a role for Fps in regulating survival in response to challenge with *Escherichia coli* (E.coli)-derived lipopolysaccharide (LPS)[67]. Using this second Fps-null model reported by Zirngibl et al. many further observations have been made which suggest not only a role for Fps in the regulation of innate immunity and inflammation, but which also point towards a mechanistic basis of how the protein does so. These observations make up the body of this thesis, and so will be described and discussed in detail in chapters 2, 3, and 4.

All studies presented in this thesis use the Fps-null (*fps*-/-) mouse model originally described by Zirngibl et al.[67]. *In vivo* studies were conducted using these mice, while *in vitro* studies were conducted using primary cells isolated from these mice. The homozygous mutant mice used were obtained by back-crossing heterozygous mutant mice to each other, and all homozygous wild type mice used as controls were obtained from these back-crossings as well. These homozygous mutant or wild type mice were then used in double homozygous breedings (i.e. *fps*-/ *fps*-/ or *fps*+/+ *fps*+/+), and Southern blots were performed on tail DNA obtained from progeny, to ensure correct genotyping. After a homozygous genotype had been confirmed for two generations, genotyping was no longer performed.
1.1.6 Role of Fps in disease

1.1.6.1 Role of Fps in inflammation

The progression and initiation of many human diseases including arthritis[74, 75], coronary heart disease[76, 77], and many types of cancer[78, 79], are affected by inflammation. The role of Fps in inflammation will be discussed further in chapters 2, 3, and 4, and how this might relate to a role for Fps in disease will be touched on in chapter 5.

1.1.6.2 Role in malignancy

Because fps/fes was originally identified in tumour-causing retroviruses[1, 2], it wasn’t hard to imagine how mutations in the fps proto-oncogene might contribute to the development of human neoplastic disease. However, for many years this connection remained elusive, until a report by Bardelli et al. in 2003 seemed to indicate that Fps might play a role in human cancer after all[80]. These researchers used high throughput sequencing to analyze the kinase domain sequences of 90 separate tyrosine kinases, in a panel of 35 colorectal cancer cell lines and 147 colorectal cancers. Their analysis found evidence of 4 separate somatic mutations in the Fps kinase domain in the cell lines investigated, and the authors went on to suggest that these mutations most likely resulted in activation of the protein, thus contributing to cancer. However, more recent work has provided biochemical, and theoretical structural analysis to suggest that all 4 mutations compromised kinase activity[81]. Furthermore, genetic analysis in mice provided evidence that inactivating or null mutations in the fps gene (fps$^{K>R/K>R}$ and fps$^{-/-}$; see sections 1.1.5.2 and 1.1.5.3) led to accelerated tumourigenesis, suggesting that Fps acts as a tumour suppressor[81]. Interestingly, Fps is the only known tyrosine kinase that has been shown to have both oncogenic, and tumour suppressive properties.
It should be noted here that since the report by Bardelli et al., two other papers have examined the rate of mutation of both fps[82], and tyrosine kinases in general in colorectal cancer[83]. Collectively, these three studies do not suggest a high rate of fps mutations in colon cancer. It remains to be seen if specific alleles/mutations of fps correlate with specific malignancies.

1.1.7 What studies on Fer might imply

Fps is the founding member of subgroup IV of the protein tyrosine kinase family; the only other member of this group being the Fer (Fes related) kinase[84]. Fer was originally identified as a 94 kDa protein recognized by antibodies raised against peptides contained in the GAG-Fps sequence, that was distinct from the 92 kDa Fps[42]. Fer was later cloned and shown to have high sequence homology, and hence predicted structural similarity to Fps[85] (Figure 1-1). Because of the homology between Fps and Fer, investigators have speculated that these two proteins may have some redundant and overlapping biological functions[36].

1.1.7.1 Fer in cytoskeletal regulation

The first piece of evidence indicating that there may be a role for Fer in regulating the cytoskeleton came from work that showed a role for Fer in mediating phosphorylation of cortactin[86], a protein known to participate in the regulation of the actin cytoskeleton[87]. Since then, several other studies have confirmed this role for Fer in regulating cortactin phosphorylation [86, 88-90]. Briefly, phosphorylation of cortactin on residues Y-421, Y-466, and Y-482 seems necessary for efficient actin disassembly [91, 92]; intriguingly, the reverse may also be true, since actin disassembly leads to cortactin phosphorylation on these same residues, a process which requires Fer[89]. These findings led to the investigation of a comparable role for Fps in
regulating the actin cytoskeleton. Preliminary work in macrophages, which do not express cortactin but do express the homologous protein HS-1, has shown that there may be a role for Fps in tyrosine-phosphorylating both HS-1 itself, and proteins associated with it (Parsons and Greer, unpublished observations); however, this study has yet to be confirmed. This implies that there may in fact be a role for Fps in actin cytoskeletal regulation, but how close to the role of Fer this is, and what the biological implications are, remains to be elucidated.

1.1.7.2 Transgenic mouse models of Fer

To investigate the biological activity of Fer, a mutant protein was produced by substituting a highly conserved aspartate residue (D743) in the kinase domain with arginine, and this resulted in the loss of Fer kinase activity[93]. Later, mice harbouring this same D>R mutation were produced (fer\textsuperscript{D>R/D>R} mutant mice), resulting in the absence of Fer kinase activity, and a reduction in the level of Fer protein itself[88]. Interestingly, much like mutant Fps mice, these mice were also viable and fertile, and displayed no overt phenotypes. However, as mentioned above, it has been proposed that Fps and Fer may have similar or redundant biological activities. Therefore, mice were bred which harbour mutations in both the fps and fer loci, by interbreeding the previously described fps\textsuperscript{K>R/K>R} and fer\textsuperscript{D>R/D>R} mutants. Surprisingly these compound mutant mice were also viable and fertile, but displayed an overall reduction in litter size from homozygous fps\textsuperscript{K>R/K>R}fer\textsuperscript{D>R/D>R} x fps\textsuperscript{K>R/K>R}fer\textsuperscript{D>R/D>R} crossings relative to wild type breeding pairs, indicating that Fps and Fer might have a redundant role in promoting fertility[94].

Unlike mutant Fps-null or fps\textsuperscript{K>R/K>R} mice, mutant fer\textsuperscript{D>R/D>R} mice did not display any defect in hematopoiesis[88]. However, later studies with these mice showed that when challenged locally with LPS, there was an increased recruitment of leukocytes to the area of inflammation[95]. Furthermore, fer\textsuperscript{D>R/D>R} mutant mice displayed an exacerbated LPS-induced intestinal epithelial barrier dysfunction[96]. These results indicated a possible role for Fer in the
regulation of inflammation. Along with previous results suggesting a role for Fps in regulating LPS-induced mortality in mice[67], these results with \( fe^{D>R/D>R} \) mice led to the investigation of the role of Fps in inflammation-induced leukocyte recruitment, which makes up a large section of chapter 3.

1.2 Background on inflammation

In 40AD, Celsus defined inflammation as consisting of “rubor et tumor cum calore et dolore,” or redness and swelling with heat and pain [De Medicina, Book III, Chapter 10]. Later in the 19th century, Virchow added a 5th tenet, \( functio laesa \), or loss of function [97]. Since that time a great deal has been elucidated about the genetic, molecular and physiological basis of inflammation, and it is now known to be the cause of, or contribute to the pathology of many diseases, including arthritis, asthma, atherosclerosis, cancer, Crohn’s disease, rheumatoid arthritis, and type 1 diabetes[98], just to name a few.

Most frequently, inflammation arises as the body’s response to an infection; however, infection and inflammation are not one in the same. Whereas infection is the invasion of the body by a foreign pathogen, inflammation is the body’s response to that invasion. Furthermore, infection is not a necessary pre-requisite to inflammation, as the latter can also be caused by burns, chemical irritants, frostbite, necrosis, allergic reaction or physical injury.

There are two main types of inflammation; acute and chronic. Acute inflammation is the initial response of the body to an injurious stimuli, such as those listed above, and involves the movement of fluids and blood cells to the site of injury. This process is relatively short lived, being initiated in minutes to hours after injury, and lasting for a period of a few days. The main cells which emigrate to the site of injury in acute inflammation are neutrophils[99]. Chronic inflammation on the other hand, is more prolonged, and can last for weeks to months, or even years. It is caused by a continual inflammatory stimulus, such as exposure to chemical agents.
(silica, for example) or autoimmune reactions (as in rheumatoid arthritis), and is characterized by persistent tissue destruction and repair, proliferation of blood vessels, and fibrosis. The main types of cells which migrate to areas of chronic inflammation are monocytes and lymphocytes[99].

The focus of this thesis is on the process of acute inflammation, and so this process will be described in more detail below, in sections 1.2.1 to 1.2.3.

1.2.1 Physiology of inflammation

As mentioned above, acute inflammation is generally due to injury or infection. This type of inflammation can be divided into four general processes, although it should be noted that this categorization is artificial in nature, as these processes overlap with each other to a greater or lesser extent. These general processes are described below, and in chapters 2, 3 and 4.

1.2.1.1 Release of mediators in response to injury

The initiation of inflammation comes when resident macrophage cells (but also dendritic, fibroblastic, mast, and endothelial cells) respond to injury by releasing a host of inflammatory mediators. Cytokines are one groups of these mediators, which can be pro-inflammatory in nature, such as tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)), or they can be anti-inflammatory in nature, such as IL-10 (Figure 1-3). Pro-inflammatory cytokines serve to promote inflammation in several ways. They induce the up-regulation of adhesion molecules on both the endothelium [100] as well as on immune cells, they activate immune cells[101, 102], and they initialize subsequent signalling cascades leading to the release of additional inflammatory mediators[100]. Furthermore, the release of molecules such as TNF-\(\alpha\) from macrophages and mast cells, or the release of histamine and serotonin from mast cells and platelets, respectively, can cause the activation and increased permeability of the vascular endothelium[98, 103].
The anti-inflammatory cytokine IL-10 serves to down regulate inflammation, by disrupting the generation of pro-inflammatory cytokines at both the transcriptional, and post-transcriptional levels[104]. The importance of this cytokine in restraining inflammation is quite apparent, as IL-10 knockout mice develop colitis and colon cancer due to chronic inflammation of the gut[105, 106].

In addition to cytokines, many lipid based molecules can also regulate inflammation. Arachidonic acid is a polyunsaturated fatty acid which is present within cell membranes, and can be metabolized into prostaglandins and leukotrienes by the COX1/2 and lipoxygenase pathways, respectively[107]. These molecules can enhance the inflammatory response, by inducing the accumulation of neutrophils during the early stages of inflammation (leukotriene B4) or by enhancing vascular permeability (prostaglandin E2)[107]. Other more recently discovered lipid metabolites termed resolvins and protectins, are also formed by both lipoxygenase pathways, or by a modified COX2 pathway in which COX2 is acetylated by aspirin[108]. These compounds are anti-inflammatory since they can block neutrophil transendothelial migration, and can also promote the clearance of cytokines, such as ligands to CCR5[108].

1.2.1.2 Changes in the vasculature

One of the first changes associated with inflammation is an increase in endothelial permeability[109], and the post-capillary venules of the microcirculation is one of the first areas in which this is observed[110]. This increased permeability comes about due to changes in the endothelial cell, initiated by transmembrane signalling events [111]. More specifically, the rearrangement of adherens junctions, the cytoskeleton, and the molecules contained within these structures, can lead to rapid changes in the structure and association of endothelial cells, and these changes allow for the transmigration of leukocytes [112, 113]. Newly recruited inflammatory cells can then pass from the circulation, through the endothelial cells, and into the surrounding
**Figure 1-3: Regulation of inflammation by secreted mediators.** Tissue injury results in the release of inflammatory mediators by white blood cells. Pro-inflammatory mediators, such as TNF-α, leukotrienes, and prostaglandins, can act to up-regulate the inflammatory response, while anti-inflammatory mediators, such as IL-10, resolvins and protectins, can act to down-regulate it. In most cases, inflammation is appropriate and balanced, resulting in the resolution of inflammation and tissue repair. Excessive release of pro-inflammatory mediators can lead to uncontrolled systemic inflammation and shock, which can result in disease and death. On the other hand, lack of appropriate inflammation allows for uncontrolled bacterial growth, which can also result in death.
tissue, where they can travel along a concentration gradient (e.g. of IL-8 or fMLP) towards the site of injury.

As well as allowing the passage of inflammatory cells, increased vascular permeability also allows for the leakage of plasma proteins into the interstitial space [103]. The movement of these proteins into the surrounding interstitial space then causes an osmotic pressure gradient, which draws fluid out of the vasculature towards the site of injury, ultimately causing swelling.

1.2.1.3 Recruitment of inflammatory cells

Another major change in the vasculature that takes place during inflammation is the up-regulation of adhesion molecules, needed to recruit inflammatory cells such as neutrophils and macrophages. This process, known as the leukocyte adhesion cascade, is a complex, multi-step process, involving the interaction of adhesion molecules on the endothelium with those on adjacent leukocytes (Figure 1-4) (reviewed in [114-116]). This process is described in more detail in chapter 3, and so will not be described further here.

1.2.1.4 Resolution of injury and tissue repair

In order to avoid excessive damage to the host, the later stages of inflammation are characterized by a resolution of the early pro-inflammatory stage. Resolution of inflammation comes about in two main ways. First, anti-inflammatory mediators such as IL-10 are released by cells such as macrophages. The presence of these molecules de-activates immune cells, thereby reducing the production of pro-inflammatory mediators[117]. Secondly, immune cells such as neutrophils, which have already carried out their role in attempting to destroy an invading pathogen, undergo apoptosis, and are subsequently phagocytosed by macrophages[118, 119]. This phagocytosis not only prevents pro-inflammatory and cytotoxic molecules from being released from necrotic neutrophils, but also serves to further stimulate the release of anti-inflammatory mediators such as IL-10 and transforming growth factor-β (TGF-β) from
Figure 1-4: Steps in leukocyte adhesion and recruitment. The figure opposite depicts the six steps involved in the recruitment of leukocytes out of the vasculature, and into surrounding tissues.

1: Leukocyte in circulation. 2: Margination: faster flowing red blood cells force leukocytes to the vessel periphery, where they come in close contact with the vessel wall. 3: Capture/tethering: upon an inflammatory stimulus, tethering is induced between the leukocyte, and the newly activated endothelium. Molecules involved: Leukocyte PSGL-1; Endothelium P-selectin. 4: Rolling: upon tethering, the leukocyte begins to roll along the endothelium, due to P-selecting/PSGL-1 bonds formed at the leading edge of the leukocyte, which are broken at the lagging edge. Molecules involved: Leukocyte PSGL-1, L-selectin; Endothelium P-selectin, L-selectin ligands. 5: Slow Rolling/Firm Adhesion: leukocyte rolling speed is diminished, until it reaches a critical point where firm adhesion can be achieved. Molecules involved: Both selectins and integrins (including CD11b). 6: Transmigration: after firm adhesion, gradients of chemoattractants such as fMLP or IL8 cause the leukocyte to migrate across the endothelium and out of the vessel towards that site of injury. Molecules involved: PECAM-1, VCAM-1, various integrins.
1.2.2 Cells involved in inflammation

1.2.2.1 Neutrophils in inflammation

The first non-resident cells recruited to the site of injury are neutrophils, which arrive at the site of injury in less than one hour. Once activated, neutrophils are stimulated to produce large amounts of cytotoxic molecules such as reactive oxygen species (ROS), or reactive nitrogen species (RNS)[100]. The principle role of these molecules is to destroy invading pathogens; however, in a hyper-inflammatory response, the amount of these cytotoxic molecules released can also cause damage to surrounding tissues, and if enough damage occurs, organ failure and death may ensue[118, 121, 122]. Neutrophils are short lived, having a half-life of approximately 5 hours when isolated from the blood; however, when encountering an inflammatory stimulus, apoptosis is avoided for 24 hours or longer[123]. Thus, in instances where there is an excess of inflammatory stimuli, the prolongation of neutrophil lifespan that results can contribute greatly to the morbidity (and possible mortality) associated with inflammation.

1.2.2.2 Macrophages in inflammation

When inflammation is triggered by a pathogen, one of the first responses of the body is to release inflammatory mediators, and the bulk of these early mediators are produced by macrophages[99, 124]. These early released mediators are mostly pro-inflammatory, and include IL-1, IL-6, IL-8, GM-CSF, interferon (IFN)-α/β/γ, and TNF-α[99]. The release of these mediators is induced through engulfment of pathogens by the macrophage, or by the interaction of molecules termed pathogen associated molecular patterns (PAMPs), with the corresponding
pattern recognition receptors (PRRs) on the macrophage. One such PAMP/PRR pair that is important in the context of inflammation is LPS and TLR4. The binding of LPS to TLR4, and the subsequent downstream signalling is outlined in more detail in chapter 2. As mentioned above, the main purpose of these pro-inflammatory molecules is to induce changes in both the vasculature and circulating blood cells, thereby inducing the migration of the latter (mostly neutrophils) to the site of injury. Therefore, the first main role of macrophages in inflammation is promotion of the response, by inducing the recruitment of inflammatory cells.

A second major function of the macrophage is to reduce the inflammatory response, and this is carried out in two ways. First, macrophages recognize and phagocytose apoptotic neutrophils, thereby preventing their necrosis and subsequent spillage of inflammatory cell contents. Secondly, macrophages also secrete anti-inflammatory mediators such as IL-10, IL-12, IL-18, and TGF-β[99, 125]. Interestingly, the ingestion of apoptotic neutrophils can lead to increased production of at least two of these cytokines, IL-10 and TGF-β[120, 122].

Macrophages also help to regulate tissue repair. Tissue repair begins with the ingestion of apoptotic neutrophils and other cellular debris, thereby “cleaning” the inflamed area[126]. These cells can then secrete (among other things) PDGF, TGF-β, FGF, and VEGF, all of which are needed at various stages of granulation, re-epithelialization, and neovascularization[99, 126]; thereby promoting tissue repair subsequent to injury.

1.2.3 Sepsis

One condition where inflammation is prominent is sepsis. This condition arises as a result of the body’s natural response to infection. Sepsis can be very mild and confined to one region of the body, such as with a tooth abscess, or can be very serious and systemic, as might result from an infection subsequent to surgery. It is estimated that severe sepsis arises in approximately 750
000 people per year in North America[127], and kills more people than prostate, breast, colon, and pancreatic cancer combined. It is the leading cause of death in the non-coronary intensive care unit, and the cost of treatment in the United States is estimated to be 17 billion dollars annually[128, 129]. Sepsis can occur in three stages: 1) Uncomplicated sepsis. This most likely occurs in millions of people every year. It is usually quite mild, and does not normally require hospital care. 2) Severe sepsis. This is more serious, and almost always requires immediate hospital care. It arises when problems occur with one or more of the vital organs, in addition to infection. 3) Septic shock. Septic shock occurs when severe sepsis is accompanied by low blood pressure, resulting in hypo-oxygenation to one or more vital organs. The mortality rate for individuals who develop septic shock is approximately 50%.

1.2.4 Use of LPS to model sepsis

One of the most popular experimental systems used to study inflammation, and used to model sepsis, is the intra-peritoneal injection of bacterial LPS into animals such as mice. This results in many of the same symptoms seen in human sepsis, as well as pathophysiological similarities including hematological alterations [130, 131], and increases in the serum levels of cytokines such as TNF-α and IL-6. However, the level of these cytokines observed in the LPS injection model is much higher than what is observed in human sepsis [130-132]. Therefore, the LPS injection model may not be the best model available to simulate sepsis as it occurs in humans. However, this model is beneficial in determining the pathophysiological response to endotoxemia and inflammation[131], as has been used in the studies described in this thesis.
1.3 Goals of this work

Before the work presented in this thesis, little was known about the role of the Fps protein in inflammation. What was known was that mice harbouring a knockout mutation in the fps gene, were more susceptible to inflammation caused by both intraperitoneal LPS challenge, and intrapediaital B. burgdorferi challenge [67, 73]. Similarly, others had shown that macrophages isolated from mice genetically altered to produce a kinase-inactive mutant Fps protein, displayed a diminished response to stimulation with inflammatory molecules such as GM-CSF and LPS[66]. These reports both suggested that a fully functional Fps protein is required to properly regulate the response to inflammation and inflammatory stimuli. This work expands on those early findings, by elucidating how the Fps protein can regulate inflammation at three separate levels. First, I examined how lack of the Fps protein alters the pathophysiology in mice, subsequent to challenge with LPS. Secondly, I showed that some of these physiological alterations were due to defects in the way in which macrophages from Fps-null mice respond to stimulation with both LPS and TNF-α. Finally, I have provided evidence that the manner in which the Fps protein regulates the response to these mediators, is through promotion of receptor internalization, possibly due to a more general role for Fps in the regulation of the cytoskeleton.
Chapter 2

The Fps/Fes kinase regulates the inflammatory response to endotoxin through down regulation of TLR4, NF-κB activation, and TNF-α secretion in macrophages.

2.1 Introduction

The *fps/fes* proto-oncogene, (hereafter referred to as *fps*), encodes a 92 kDa Fps protein which belongs to subgroup IV of the non-receptor protein tyrosine kinases [43, 84]. The ubiquitously expressed 94 kDa Fer protein is the only other known member of this subgroup of kinases [42, 84, 85]. In the adult, Fps is expressed in hematopoietic cells of the myeloid lineage, including macrophages, neutrophils, mast cells, platelets, and red blood cells, as well as in certain neuronal and epithelial cells. In contrast, during development Fps is expressed in all three germ layers [46, 48, 133] (reviewed in [36]).

The innate immune system defends against invading pathogens by initiating an inflammatory response, and this requires the activation of key cell types, including macrophages, neutrophils and mast cells [102]. Mouse knockout models have provided evidence for the involvement of Fps [67] and Fer [95] in the regulation of innate immune responses. Fps-null mice displayed increased mortality in response to intra-peritoneal challenge with the endotoxin LPS [67], and Fer-deficient mice displayed increased leukocyte recruitment at sites of localized LPS challenge [95] and enhanced intestinal barrier dysfunction in response to LPS[96]. LPS is a component of the cell membrane of gram negative bacteria, which is recognized by cells of the innate immune system. The biological response to LPS is mediated by a receptor complex composed of CD14, MD2, LBP, and Toll-like receptor (TLR) 4. TLR4 is a trans-membrane receptor, belonging to the TOLL/IL-1 receptor family [134], and when stimulated, this receptor initiates an intracellular signaling cascade that results in the activation of extracellular signal regulated kinase (Erk), c-Jun N-terminal kinase (Jnk), p38, Akt, and nuclear factor-κB (NF-κB).
Activation of NF-κB is a two-pronged process, whereby phosphorylation-induced degradation of its bound inhibitor, inhibitor of κB (IκB), uncovers an NF-κB nuclear localization sequence, freeing it to move into the nucleus. As well, post translational modifications of NF-κB such as phosphorylation of the p65/RelA subunit, causes a more robust and prolonged transcription of its target genes (reviewed in [137]). Once activated, these signaling molecules, particularly NF-κB, induce the transcription of pro-inflammatory mediators such as TNF-α [138].

Recently, it has been demonstrated that signaling by TLR4 is regulated in part by endocytosis [139]. More specifically, inhibition of TLR4 internalization leads to an increase in NF-κB activation, and this process is dependent on dynamin and clathrin [139].

We have previously shown evidence of a defective innate immune response to LPS in mice lacking either Fps [66, 67], or the closely related Fer kinase [95, 96]. However, the precise cellular and molecular bases for these defects have not yet been established. We now report an increase in circulating TNF-α levels in LPS-challenged Fps-null mice. This increase in pro-inflammatory TNF-α strongly correlates with the hypersensitivity of Fps-null mice to LPS [67], and therefore suggests that Fps might play an important role in regulating the innate immune response to endotoxin through controlling production of this key cytokine. We also describe an enhanced activation of the LPS-induced NF-κB signaling pathway in Fps-null macrophages, which provides a likely mechanistic basis for the enhanced TNF-α secretion by macrophages. We go on to demonstrate that down regulation of TLR4 is defective in Fps-null macrophages, which may be the cause of the enhanced NF-κB signaling. This defect in TLR4 internalization may reflect a more general role for Fps in internalization, since Fps-null macrophages also showed defects in internalization of transferrin and uptake of E.coli. Defective TLR4 internalization, causing enhanced LPS-induced activation of NF-κB, and leading to elevated production of TNF-α, provides a highly plausible physiological explanation for the increased susceptibility of Fps-null mice to endotoxic shock.
2.2 Materials and Methods

2.2.1 ELISA assays

For in vivo assays, age-matched male mice were weighed one day before intraperitoneal (i.p.) injection with 7mg/kg LPS (E.coli, serotype 055:B5, Sigma), and sacrificed at different times by chloroform inhalation. Chest cavities were opened, and blood was removed by cardiac puncture with a 1 mL syringe fitted with a 26 gauge needle using 0.3% tri-sodium citrate as anti-coagulant. Blood was centrifuged for 1 minute at 18000 g. Plasma was collected and frozen at -80°C. For in vitro assays, conditioned media from LPS-stimulated peritoneal macrophages at the indicated times (see below, and Figure 4), was collected and frozen at -20°C. Enzyme-linked immuno-sorbent assay (ELISA) assays were performed using OptEIA Mouse IL-10, and TNF-α sets from BD Biosciences, according to manufacturer’s instructions.

2.2.2 Peritoneal lavage

Mice were euthanized as above, and peritoneal lavage was performed twice with 5 mL of pre-warmed lavage media (RPMI 1640 with 10 mM HEPES, 5 mM EDTA, 10 U/mL Heparin, 1% Pre antibiotic-antimycotic (a.a){GIBCO}, 50 µM α-monothioglycerol). Cells were pelleted and resuspended in 5 mL of erythrocyte lysis buffer (154 mM NH₄Cl, 10 mM KHCO₃, 100 µM EDTA) for 5 minutes at 4°C to lyse red blood cells. The remaining cells were then pelleted and resuspended at 1 x 10⁶ cells/mL, and 5 x 10⁵ cells/mL (for assays done in 6 and 12 well plates, respectively) in culture media (RPMI 1640 with 5% FBS, 1% a.a., 50 µm α-monothioglycerol, 1 mM HEPES, 2 mM glutamine).

2.2.3 Stimulation of resident peritoneal macrophages

Cells collected by peritoneal lavage were plated in 12 well plates (5 x 10⁵ cells per well), and 2 to 3 hours later, non adherent cells were washed off using sterile phosphate buffered saline.
Cells were then allowed to incubate overnight, and the next day adherent cells were stimulated with LPS at the indicated concentrations and times. To stop reactions, media was removed, and plates were placed on ice with 1 mL of Tris buffered saline containing 100 µm sodium orthovanadate (TBS-V) per well. Soluble cell lysates were prepared by aspirating TBS-V and scraping cells into 250 µl of kinase lysis buffer (KLB), containing protease and phosphatase inhibitors (20 mM Tris-HCl {pH 7.5}, 150 mM NaCl, 1 mM EDTA, 1% {vol/vol} Nonidet P-40, 0.5% {vol/vol} sodium deoxycholic acid, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 100µM sodium orthovanadate, 100 µM phenylmethylsulfonyl fluoride) using a rubber policeman. Lysates were then spun for 10 minutes at 4°C and 14000 g, soluble material added to clean tubes containing 6X SDS protein sample buffer, heated to 100°C for 5 minutes, passed through a P-200 pipette tip several times to shear high molecular weight DNA, and spun briefly at 14000 g. Lysates were either frozen at -20°C, or run immediately on 7.5% or 11% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Whole cell lysates were obtained by scraping cells into 1X SDS protein sample buffer directly, and frozen without centrifuging. Proteins were transferred by semi-dry blotting to Immobilon-P membrane (Millipore), blocked with either 5% milk powder in TBS-Tween, or 5% BSA in TBS-tween, and probed with the following primary antibodies: rabbit anti-phospho-p44/42 (pERK1/2), rabbit anti-phospho-p38, rabbit anti-p38, rabbit anti-IκBα, rabbit anti-phospho-p65(Ser536)NF-κB (Cell Signalling Technology), rabbit anti-p44/42 (ERK1/2) (Santa Cruz), rabbit anti-Fps/Fer antibody [48].

2.2.4 Flow cytometry

For Figures 2-4 and 2-5A, adherent cells’ FcγII/III receptors were blocked by incubation with conditioned media from 2.4G2 hybridoma cells (ATCC# HB-197) for 5 minutes on ice. Cells were then incubated with either 2 µg/mL phycoerythrin (PE)-conjugated rat anti-mouse TLR4/MD2 (eBioscience, San Diego California) for 15 minutes on ice, post LPS stimulation.
(Figure 2-4), or 40 µg/mL Alexa594 Transferrin, or isotype control (Molecular Probes, Eugene, Oregon), for 30 min. at 37°C (Figure 2-5A). Cells were then washed with ice-cold PBS, fixed for 15 minutes with formaldehyde/zinc fixative (Electron Microscopy Sciences, Fort Washington PA), and scraped into ice-cold PAB (PBS containing 3% bovine serum albumin [w/v] and 1% sodium azide [w/v]) for analysis by flow cytometry. For Figure 2-5B, cells from the peritoneal cavity were collected by lavage. After lysing erythrocytes, cells were resuspended at 1x 10^6 cells/mL in RPMI and incubated with 5 x 10^5/mL green fluorescent protein (GFP)-expressing E.coli, for 30 minutes at 37°C. Cells were then washed, resuspended in PAB, placed on ice, and incubated with 0.1 µg/mL PE-conjugated rat anti-mouse F4/80 antibody to stain for macrophages. Cells were then washed, fixed as above, and analysed by flow cytometry.

2.2.5 Mice

All animals used in this study were inbred SvJ/129 mice, between 7 and 12 weeks old, and all experiments were carried out according to the guidelines of the Canadian Council on Animal Care, and with the approval of the institutional animal care committee.

2.2.6 Statistics

All error bars represent standard error of the mean. All reported statistical values were calculated using the Student’s T-test.

2.3 Results

2.3.1 Elevated plasma levels of TNF-α in Fps-null mice challenged with LPS.

We have previously shown that Fps-null mice experience a higher rate of mortality when challenged with an i.p. injection of LPS corresponding to the lethal dose (LD)_{50} for wild type mice [67]. Since production of inflammatory and anti-inflammatory cytokines plays a key role in
regulating the endotoxin response, we examined plasma TNF-α and IL-10 levels in mice challenged with an i.p. injection of LPS. ELISA analysis demonstrated that Fps-null mice had statistically significant increases in circulating TNF-α at 1 hr post LPS injection (5277 ± 832 pg/mL vs. 8025 ± 283 pg/mL, p=0.014, Fig. 2-1, top panel). At 2 hours post injection, Fps-null mice also displayed an apparent decrease in IL-10, (1432 ± 166 pg/mL vs. 875 ± 216 pg/mL, p=0.092, Fig. 2-1, bottom panel), although this difference did not reach statistical significance. These results suggest a skewing in the balance of pro- and anti-inflammatory cytokines towards a heightened inflammatory state in Fps-null mice, which correlates with the increased mortality observed previously [67].

2.3.2 Resident peritoneal macrophages from Fps-null mice secrete increased amounts of TNF-α in response to LPS stimulation ex vivo.

We next sought to examine whether macrophages could be one of the cell types responsible for the increased levels of circulating TNF-α in LPS-challenged Fps-null mice. To address this possibility, resident peritoneal macrophages were harvested, stimulated with LPS, and TNF-α levels in the culture media were measured by ELISA (Fig. 2-2). At one, two, and four hours post stimulation, the level of TNF-α in the media of Fps-null cultures was higher than in the wild type cultures, and by six hours post stimulation, it was 2.5 times higher in Fps-null compared to wild type (Fig. 2-2; p<0.05 for 1, 4, 6 hour time points). This suggested that macrophages were at least partly responsible for the in vivo differences observed between Fps-null and wild type mice subsequent to an LPS challenge.

2.3.3 Prolonged IκB-α degradation and enhanced NF-κB phosphorylation following LPS stimulation of Fps-null macrophages.

We next looked for differences in downstream signaling. Immunoblotting analysis of a
Figure 2-1: Fps-null mice display a hyper-inflammatory phenotype as assessed by measuring plasma cytokine levels post-challenge with LPS. Mice were injected i.p. with 7 mg/kg LPS. At the indicated time points afterwards, mice were euthanized, and whole blood was collected by cardiac puncture. Blood was centrifuged, and plasma collected and analyzed by ELISA for TNF-α (top panel) and IL-10 (bottom panel). For all time points, n = 4 or 5.
Figure 2-2: Resident peritoneal macrophages from Fps-null mice secrete increased levels of TNF-α in response to stimulation with LPS. Resident peritoneal macrophages were isolated by lavage and allowed to adhere to tissue culture dishes. Cells were stimulated for the indicated time points with 270 ng/mL LPS. After stimulation culture media was removed and in vitro TNF-α levels were analyzed by ELISA. For all time points, n=3.
number of signaling molecules was performed on peritoneal macrophages after in vitro challenge with LPS. This analysis showed a prolonged period of degradation of IκB-α in Fps-null macrophages as compared to wild type counterparts (Fig. 2-3A). IκB-α levels returned to near-basal levels between one and two hours after LPS-challenge in wild type cells, while IκB-α levels remained depleted at two hours in Fps-null cells. We also examined the phosphorylation status of ERK and p38, two other kinases known to be activated downstream of LPS signaling; however no difference in the phosphorylation status of either kinase was found between wild type and Fps-null macrophages (Fig. 2-3A).

The observed difference in LPS-induced IκB-α degradation/recovery kinetics (Fig. 2-3A) suggested prolonged or enhanced NF-κB activation in Fps-null macrophages. This was assessed by immunoblotting analysis using a phospho-specific antibody against serine 536 of the p65/RelA subunit of NF-κB. These analyses also indicated enhanced LPS-induced NF-κB activation in Fps-null macrophages as compared to wild-type. Phosphorylation of p65 was increased in Fps-null relative to wild type macrophages at 15 minutes post LPS stimulation. The levels of p65 phosphorylation declined between 15 and 30 minutes in both genotypes, and continued to decline in wild type cells back to pre-LPS challenged levels by 120 minutes. However, in Fps-null macrophages, p65 phosphorylation levels persisted at the same level between 30 and 120 minutes (Fig. 2-3B). Interestingly, this correlated with the previously observed kinetics of IκB-α degradation/recovery (Fig. 2-3A). Taken together, these observations suggest a role for Fps in both LPS-induced NF-κB activation and inactivation.

2.3.4 Prolonged surface expression of TLR4/MD2 following LPS stimulation of Fps-null macrophages.

In attempts to explain the molecular basis for the observed difference in LPS signaling, we next assessed surface expression of the LPS receptor, TLR4, before and after LPS stimulation
Figure 2-3: Resident peritoneal macrophages from Fps-null mice show prolonged degradation of IκB-α and increased phosphorylation of NF-κB, in response to stimulation with LPS. Resident peritoneal macrophages were isolated by lavage and allowed to adhere to tissue culture dishes. Cells were stimulated for the indicated time points with 270 ng/mL LPS (+) or vehicle control (-). After removing media for ELISA analysis, cells were lysed, and soluble cell lysates (SCLs) were resolved on either 7.5% or 11% SDS-PAGE gels, transferred to membranes and probed with the indicated antibodies.
Figure 2-4: Resident peritoneal macrophages from Fps-null mice display a defect in down-regulation of TLR4/MD2 in response to LPS stimulation. Resident peritoneal macrophages were isolated by lavage and allowed to adhere to tissue culture dishes. Cells were stimulated for the indicated time points with 270 ng/mL LPS. Following stimulation, cells were washed with ice-cold TBS-V, and labeled with a PE-tagged α-TLR4/MD2 antibody. Cells were then scraped and analyzed by flow cytometry to measure the relative surface expression of TLR4/MD2. For all time points, n=6.
of cultured peritoneal macrophages. Flow cytometry analysis using an antibody specific for TLR4/MD2 showed rapid loss of surface-accessible receptor in LPS-treated wild type cells, with approximately 25% remaining after 5 minutes (Figure 2-4). In contrast, greater than 60% remained on the surface of Fps-null macrophages after 5 minutes (27% WT vs. 63% fps⁻/⁻; p=0.029), and there was little apparent further reduction at 15 minutes (28% WT vs. 52% fps⁻/⁻; p=0.030). These data suggested that Fps is participating in the process by which TLR4 is internalized from the surface of macrophages after LPS exposure.

### 2.3.5 Fps-null macrophages show reduced uptake of both transferrin and E.coli.

Internalization of growth factor and cytokine receptors [140], reorganization of cell-cell and cell-matrix receptors[141], and phagocytosis [142], all involve dynamic cytoskeletal reorganization. A number of studies have suggested that Fps [15, 16, 35, 61] and the related Fer kinase [89, 90, 143-147] might play roles in cytoskeletal remodeling. We therefore considered the possibility of a more general receptor internalization defect in Fps-null macrophages. Indeed, flow cytometry analysis of Alexa594-labelled transferrin showed that uptake of transferrin by wild type macrophages was 3.5 times higher than in Fps-null cells (Fig. 2-5A middle columns, p<0.001). In order to differentiate between transferrin on the cell surface and that which had been internalized, a stripping method (see material and methods) was used to remove any transferrin bound to the cell surface. This approach revealed a defect in transferrin internalization in Fps-null macrophages, with wild type cells internalizing 2.3 times more than their Fps-null counterparts (Fig. 2-5A, right side columns, p=0.018).

Transferrin[148] and TLR4[139] are both internalized by receptor mediated mechanisms. Therefore, we explored the possibility that the defects in receptor internalization in Fps-null cells might represent a broader defect that was not restricted to receptor-mediated endocytosis. Wild type and Fps-null macrophages were incubated with E.coli expressing enhanced GFP (EGFP) for 30 min., and then examined by flow cytometry. In this analysis, 41.6% of wild type cells stained
Figure 2-5: Resident peritoneal macrophages from Fps-null mice display a reduced internalization of transferrin, and reduced uptake of *E. coli*. Resident peritoneal macrophages were isolated by lavage and either allowed to adhere to tissue culture dishes (A) or used directly (B). A: Cells were stimulated with 40 µg/mL Alexa594-labelled transferrin for 30 min. Transferrin incubated cells were then either surface-stripped or not, using a solution of 0.5 M NaCl and 0.2 M acetic acid. Cells were then scraped and analyzed by flow cytometry. For all time points, n=4. B: Cells were incubated with GFP-expressing bacteria, for 30 min. Cells were then incubated with α-F4/80 antibody to detect macrophages, washed, and analyzed by flow cytometry. For all time points n=3; for bolded quadrants, n=0.071 WT vs. Fps-null.
positive for both F4/80 antigen and GFP, while in the Fps-null cells, only 27.1% were double positive (Fig. 2-5B, p=0.071 wild type vs. Fps-null). This was consistent with Fps having a broader role in internalization, and not specifically in receptor-mediated endocytosis.

2.4 Discussion

Previous studies have utilized knockout mice to establish a role for Fps in the regulation of innate immunity and inflammation [67, 73]; however, they failed to provide a mechanistic basis for Fps’s participation in these processes. Here, we further the understanding of Fps’s role in inflammation and innate immunity, by showing that Fps-null mice displayed an increase in plasma TNF-α in response to an in vivo LPS challenge. This effect was also observed ex vivo in isolated Fps-null peritoneal macrophages treated with LPS. In addition, we provided evidence that the heightened TNF-α secretion was likely due to an enhanced activation of the NF-κB pathway in response to LPS stimulation, which might be a consequence of the failure of Fps-null macrophages to internalize TLR4. Finally, we showed a role for Fps in internalization of both transferrin and E. coli, suggesting a more general role for Fps in internalization.

In innate immune responses to infection with gram negative bacteria, recognition of LPS by macrophages and other cells results in the release of pro-inflammatory cytokines such as TNF-α, followed by the release of anti-inflammatory cytokines such as IL-10. Balanced production of these and other key mediators of inflammation is essential to a controlled innate immune response. Fps-null mice displayed significantly higher levels of TNF-α in their plasma after LPS challenge (Fig. 2-1; p=0.014). Previous work has shown that administration of a TNF-α antibody 6 hours prior to LPS injection in mice, reduces mortality by approximately 50% [149], and that mice injected with recombinant TNF-α display many of the same pathophysiological symptoms as those injected with LPS [150], thus establishing TNF-α as a pivotal mediator of the effects of endotoxin in vivo. Therefore, our observation that plasma TNF-α levels induced by
LPS challenge were nearly twice as much in Fps-null mice than wild type mice provided a highly plausible physiological explanation for the differences in LPS-induced mortality described previously in these animals [67]. We also observed a 64% decrease in the peak levels of IL-10 in the plasma of Fps-null mice (Fig. 2-1). Although this difference did not reach statistical significance, it was consistent with a general defect in the balance of pro- and anti-inflammatory cytokine levels. Taken together, these results suggest that defective regulation of cytokine release by macrophages is a major factor in the higher susceptibility of Fps-null mice to challenge with LPS.

Among known Fps-expressing cell types, macrophages represented an excellent candidate for the cell type responsible for the increased production of TNF-α during inflammation. Furthermore, they are also an important source of IL-10 during the resolution phase [125]. This was substantiated by the observation that cultured peritoneal Fps-null macrophages produced significantly increased LPS-induced TNF-α levels at one, four, and six hours in vitro (Fig. 2-2), suggesting that this cell type was at least partially responsible for the increased in vivo levels of TNF-α in LPS-challenged Fps-null animals. Since Fps is also expressed in other innate immune cells, including mast cells and neutrophils, it should be appreciated that these might also contribute to the observed in vivo hyper-inflammatory phenotype; however, due to the observed role for Fps in TNF-α secretion in cultured peritoneal macrophages (Fig. 2-2), it was clear that the macrophage response was important to the phenotype observed in vivo.

The observed differences in IκB-α degradation suggested a prolonged time over which NF-κB would be localized to the nucleus in Fps-null macrophages. This would lead to enhanced TNF-α transcription, and eventually an increase in secretion of TNF-α into the culture media. Indeed, ELISA analysis provided direct evidence for accumulation of greater TNF-α levels in Fps-null cultures after LPS challenge (Fig. 2-2). However, an optimal NF-κB response also involves post translational modifications including phosphorylation the p65/RelA subunit on
Ser529 and Ser536 (reviewed in [137]). Interestingly, we observed a more robust phosphorylation of p65/RelA on Ser536 in Fps-null macrophages at 15 minutes after LPS stimulation compared to wild type cells (Fig. 2-3B). This provided additional evidence for an increase in the transcriptional activation status of NF-κB in Fps-null macrophages. An intriguing possibility is that this increase in NF-κB signaling might also exist downstream of TNF-α stimulation. This is intriguing since TNF-α can act back on these cells in an autocrine manner [151] and cause degradation of IκB-α (reviewed in [152]); therefore the increased TNF-α secretion by Fps-null macrophages (Fig. 2-2) could have contributed to the prolonged degradation of IκB-α seen in these cells (Fig. 2-3A), and participation in TNF-α signaling would give Fps a two-tiered role in the LPS response.

At first glance, there seem to be discrepancies between the present results and those that were described in a previous paper (Zirngibl et. al. 2002, [67]), since the previous paper reported no observed differences between wild type and Fps-null cells with respect to IκB degradation. However, there are two key differences in how the experiments were conducted, which might account for these differences. First of all, the previous paper used bone marrow-derived macrophages, while this study used resident peritoneal macrophages. Differences in the production of cytokines such as TNF-α, between various sub-types of macrophages is well established [153]. We therefore speculate that some differences in signalling might also be due to the use of two different sub-types of macrophages. Secondly, in the previous paper, bone marrow derived macrophages were stimulated at an LPS concentration of 1 µg/mL, which is four times higher than the 270 ng/mL used here. We observed that 270 ng/mL LPS corresponds to a concentration which induces near maximal stimulation of macrophages (data not shown); therefore, the use of a LPS concentration which is nearly 4 times higher in the previous paper might have masked some of the differences we describe here.

Macrophages isolated from Fps-null mice, did not internalize TLR4 to the same extent as
their wild-type counterparts, at both 5 and 15 minutes post stimulation with LPS. This observation was consistent with our other data, since major defects in the NF-κB pathway were observed at 15 minutes post LPS challenge (Fig. 2-3). Furthermore, our results agree with the observations that TLR4 has been shown to generate inflammatory signals from the cell surface [154], and that endocytosis of this receptor is necessary in limiting LPS-induced NF-κB activation [139]. However, as is shown in figure 2-3A, the observed defect in endocytosis of TLR4 does not appear to affect phosphorylation of either p38 or ERK. This result is consistent with other reports describing an effect of endocytosis on some, but not all signalling pathways downstream of the same stimulus [155-157].

Interestingly, the internalization defect observed in Fps-null macrophages was not specific for TLR4, but also extended to internalization of transferrin and uptake of \textit{E.coli} (Fig. 2-5A, B). We therefore propose that Fps’ role in LPS signaling might be at the level of receptor internalization and/or intracellular receptor-complex trafficking, possibly by regulating cytoskeleton reorganization processes required for these events. This is an attractive hypothesis for a number of reasons. Firstly, TLR4 signaling is not necessarily connected to the internalization of bacteria (reviewed in [158]), and therefore the observed participation of Fps in both suggests a less specific role. However, both processes are dependent on actin cytoskeleton reorganization, and the Fps-related Fer kinase has been shown to be connected to actin cytoskeleton function through regulation of cortactin phosphorylation [86, 88-90]. Since Fps and Fer are highly homologous and share the same domain structure[85], it is thought that these kinases may have similar or redundant roles within the cell, and so Fps might also contribute to regulation of the actin cytoskeleton. Secondly, recent work by Laurent \textit{et. al.} showed a role for human Fps in the regulation of the tubulin cytoskeleton [15], which is also important for both phagocytosis [159], and endocytosis (reviewed in [160]). Also, Fps partially co-localized with Rab5B, Rab7, and a marker of the trans-golgi network, suggesting a role in vesicular trafficking [20], and TLR4 has been shown to traffic to lysosomes for degradation[139].
In summary, this study establishes a biological role for Fps in the regulation of innate immunity through control of TNF-α production by macrophages, which might explain why its absence negatively affects the survival of mice challenged with LPS[67]. While the precise molecular basis of this function is still unknown, we propose that it is due to a defect in internalization of TLR4, which leads to more pronounced and sustained activation of NF-κB. Finally, since internalization of transferrin and uptake of *E.coli* are also affected by the absence of Fps, this might suggest a more general role for Fps in modulation of the cytoskeleton.
Chapter 3
The Fps/Fes kinase regulates leukocyte recruitment and extravasation during inflammation.

3.1 Introduction

The \textit{fps/fes} proto-oncogene, (hereafter referred to as \textit{fps}), encodes a subgroup IV non-receptor protein-tyrosine kinase [43, 84]. The only other known member of this subgroup of kinases is the ubiquitously expressed Fer protein [42, 84, 85]. Fps expression has been described in hematopoietic cells including monocytes/macrophages, neutrophils, mast cells, platelets, and erythrocytes; as well as in some neuronal, epithelial and vascular endothelial cells. In contrast, during development Fps is expressed in all three germ layers [46, 48, 133] (reviewed in [36]).

The Fps and Fer kinases consist of a C-terminal kinase domain, a central SH2 domain and an N-terminal Fps/Fer/CIP4 homology (FCH) domain associated with three coiled-coiled domains [36]. This unique N-terminal structure distinguishes Fps and Fer from all other members of the protein-tyrosine kinase family, and it is thought to mediate associations with phospholipid components of membranes as well as the cytoskeleton [15, 16, 22].

The body responds to invading pathogens by initiating an inflammatory response, and this requires the activation of key cell types, including macrophages and neutrophils, which make up the bulk of the body's inflammatory cells [102]. Of great importance in this response is the movement of leukocytes out of the blood, and into surrounding tissues towards the site of injury or infection. The leukocyte does this through an ordered series of events involving interactions between the inflammatory cells and the endothelium. The first step in leukocyte recruitment involves the interaction between selectins on the activated endothelium, and their ligands on the leukocyte, which mediates leukocyte rolling along the endothelium. The two endothelial selectins primarily responsible for this initial rolling are E- and P-selectin. Evidence for this is demonstrated by the near total lack of leukocyte rolling in mice engineered to harbor a double
knockout for these two selectins [161, 162]. Furthermore, there is an observed defect in leukocyte rolling in the P-selectin single knockout mice, and a defect in slow rolling in E-selectin knockouts [163]. The primary leukocyte ligand for P-selectin [164-166], and to a lesser extent E-selectin [167], is CD162, or P-selectin glycoprotein ligand 1 (PSGL-1). Consistent with its role in binding endothelial P-selectin, mice deficient for PSGL-1 show reduced leukocyte rolling upon TNFα-induced inflammation in the cremaster muscle, and a reduced thioglycollate-induced neutrophil influx into the peritoneal cavity; and the magnitude of these defects are similar to those observed in P-selectin knockout mice [168]. Subsequent to rolling, leukocytes must next achieve firm adhesion along the inflamed vessel before transmigration can take place. One of the proteins involved in this process is the αMβ2 integrin, also known as Mac-1 or CD11b/CD18. A counter ligand for CD11b/CD18 on the endothelium is intercellular cell adhesion molecule 1 (ICAM-1). CD11b/CD18 has been reported to participate in both adherence and transmigration of leukocytes [169].

Due to the expression of Fps and Fer in macrophages, neutrophils and the vascular endothelium, we have explored the possibility of a role for these tyrosine kinases in regulation of the innate immune response. We have previously reported increased mortality in Fps knockout (fps−/−) mice in response to LPS challenge in vivo [67], which was at least partially due to increased in vivo levels of TNFα caused by enhanced NF-κB signalling in macrophages [170]. Mice deficient in the Fer kinase also displayed increased LPS-induced neutrophil adhesion to venules and extravasation in the cremaster muscle [95] and the small intestine submucosa [96].

Here we show for the first time that Fps-null mice display increased inflammation as measured by neutrophil rolling, adhesion, and extravasation in cremaster venules subsequent to LPS challenge, with concomitant defects in the hemodynamic parameters of these same vessels. Using reflected light oblique transillumination (RLOT) intravital microscopy, we further show that the rate at which leukocytes cross the cremasteric endothelial barrier is increased in Fps-null mice. There was also an increase in the number of neutrophils recruited to the peritoneal cavity of
Fps-null mice subsequent to thioglycollate challenge. We also observed a prolonged retention of surface PSGL-1 on neutrophils from Fps-null mice subsequent to stimulation with LPS. This latter observation is mechanistically consistent with the observed increases in leukocyte rolling, adhesion and recruitment observed in inflamed tissues of Fps-null mice. Collectively, these observations provide novel evidence implicating Fps in the regulation of innate immunity and the vascular response to inflammation.

3.2 Materials and Methods

3.2.1 Cremaster Surgical Preparation and Intravital Microscopy

Intravital microscopy was performed essentially as described by McCafferty et. al.[95], with a few exceptions. Briefly, mice were injected subcutaneously with LPS (0.05 µg/kg) into the left side of the scrotum. A 65 mg/kg I.P. injection of Somnotol was used as anesthetic, 4-0 surgical silk was used for suturing the cremaster muscle, and the suffusate passed over the externalized cremaster muscle was a buffered modified Krebs solution. LPS was from E.coli serotype 055:B5 (Sigma). Three hours later, mice were anesthetized and the contra-lateral cremaster muscle was externalized for intravital video-microscopy recording. Leukocyte rolling, adherence, and emigration, were quantified in venules of approximately 30 micron diameter. Rolling flux was calculated as the number of cells rolling past a designated point in the vessel per minute. For assessment of wall shear rate, red blood cell (RBC) velocity was divided by the vessel diameter, and multiplied by 8000. For experiments measuring transmigration rates, we used RLOT microscopy, which makes use of the optical interference phenomena generated by oblique transillumination in conjunction with intravital microscopy. This method utilizes subtle gradients of refractive indices within the tissues for enhanced image contrast as designed by Mempel et. al [171]. Briefly, the microscope was retrofitted with a 700 nm +/- 20 nm band pass filter in the light path of a 100 W halogen lamp, which illuminated the specimen via 50/50 beam splitter. Beneath the tissue was placed a 24 mm² glass cover slip, painted with aluminum to create a
mirror, oriented at an angle of approximately 10 or 15 degrees relative to the stage for the 20X and 40X objectives, respectively. Extravasating leukocytes were visualized by RLOT video microscopy in cremaster muscle post capillary venules of approximately 30 micron diameter. Transmigration rates were calculated as the elapsed time from firm adhesion to tissue migration.

3.2.2 Flow cytometric analysis of adhesion molecule expression on peripheral blood leukocytes

Age-matched mice were sacrificed by inhalation of chloroform. Chest cavities were opened, and blood was removed by cardiac puncture with a 1 mL syringe fitted with a 26 gauge needle using 0.3% tri-sodium citrate as anti-coagulant. Blood was then stimulated with LPS at the indicated times and concentrations. Samples were washed twice with, and resuspended in 300 µL of cold PAB (PBS with 0.3% [w/v] BSA, 0.1% [w/v] sodium azide). Samples were incubated with 1.5 µg/mL FITC-conjugated CD11b antibody or 1.0 ug/mL PE-conjugated PSGL-1 (CD162) antibody (BD Pharmingen, Mississauga, ON), for 15 minutes on ice and washed twice with PAB. Samples were then incubated for 3 minutes at 4°C in ACK buffer (154 mM ammonium chloride, 10 mM potassium bicarbonate, 100 µM EDTA) in order to lyse red blood cells. Finally, cells were washed and resuspended in 500 µL of PAB, vortexed, combined with 500 mL of paraformaldehyde-zinc fixative (Electron Microscopy Sciences, Fort Washington, PA), and analyzed by flow cytometry. Neutrophils were gated using forward and side scatter; monocytes/lymphocytes were gated using forward and side scatter, and monocytes were further separated on the basis of CD11b expression.

3.2.3 Leukocyte Recruitment Assay

Mice were injected I.P. with 1 mL of 4% thioglycollate in PBS, using a 1 mL syringe fitted with a 26 gauge needle. Four hours after injection, peritoneal lavage was performed twice
per mouse with 5 mL of pre-warmed lavage media (RPMI 1640 with 10 mM HEPES, 5 mM EDTA, 10 U/mL Heparin, 1% [vol/vol] antibiotic-antimycotic [GIBCO], 50 μM α-monothioglycerol). Following lavage, cells were washed, counted on a Beckman Coulter Z1 particle counter, and resuspended at 1 x 10^6 cells/mL in PAB. Next, 200 μL was transferred into 4 mL snap-cap tubes with a final concentration of 0.2 μg/mL PE-conjugated Ly6G antibody (eBioscience, San Diego, CA) and 1.5 μg/mL fluorescein isothiocyanate (FITC)-conjugated CD11b antibody, as indicated, for 15 minutes on ice. Cells were then washed and resuspended in 500 μL of PAB, vortexed, added to 500 μL of formaldehyde-zinc fixative, and analyzed by flow cytometry as indicated.

3.2.4 Mice

All animals used in this study were inbred SvJ/129 mice, between 7 and 12 weeks old. The Fps-null strain of mice was previously described [67]. All experiments were carried out according to the guidelines of the Canadian Council on Animal Care, with the approval of the institutional animal care committee.

3.2.5 Statistics

All error bars represent standard error of the mean. For Figures 3-1, 3-2 and 3-5, and Table 3-1, statistical values were calculated by two-way repeated measures analysis of variance (ANOVA) using Graph Pad Prism 4 software. All other reported statistical values were calculated using the Student’s T-test. All statistical values reported represent a comparison of wild type vs. fps⁻⁻. 
3.3 Results

3.3.1 Fps regulates the local inflammatory response to challenge with LPS.

Upon localized LPS challenge, leukocytes and the endothelium each follow an ordered sequence of progressive activation events, ultimately resulting in emigration of activated leukocytes out of the vessels into the surrounding tissue [114]. During this transmigration process, inflammatory cells initially loosely tether to the vessel, allowing them to roll along its surface. Rolling gradually slows, until a firm adhesion and subsequent emigration of the leukocyte across the vessel wall into surrounding tissue is achieved [114]. In order to explore the potential role of Fps in LPS-induced leukocyte transmigration, we compared the in vivo response to localized LPS challenge in wild type and Fps-null mice using a cremaster muscle intravital microscopy model [95].

Rolling flux (the number of cells rolling past a designated point in the vessel per unit time) is directly related to the number of rolling leukocytes, but inversely related to their rates of rolling, which is a function of their degree of activation. Rolling flux thus serves as a useful indicator of the state of the inflammatory response, with lower values corresponding to higher degrees of inflammation. Leukocytes in Fps-null mice had a lower rolling flux at 3 and 4 hours post injection compared to those in wild type mice, and a two-way ANOVA analysis across the 3, 3.5 and 4 hour time points indicated a statistically significant reduction in rolling flux in Fps-null mice (Figure 3-1A; P=0.005). Leukocyte rolling velocities were significantly higher in wild type relative to Fps-null mice at 3, 3.5, and 4 hours post LPS injection (Figure 3-1B; P=0.008). The number of cells rolling along a 100µm section of vessel was calculated by dividing the rolling flux by the rolling velocity. At 3 hours post LPS challenge, wild type and Fps-null vessels contained the same number of rolling cells. However, by 3.5 hours post challenge, the number of rolling cells in wild type vessels had decreased dramatically, suggesting that the inflammation was beginning to resolve. In contrast, a reduction in rolling leukocytes was not observed in Fps-null vessels until 4 hours post LPS injection (Figure 3-1C), indicating a more prolonged
inflammatory state in the Fps-null mice.

The number of fully adherent leukocytes per 100 μm of vessel was also greater in Fps-null mice at all time points examined, which again suggested that these mice were in a hyper-inflammatory state as compared to wild-type animals (Figure 3-2A; P=0.004). Consistent with these observed decreases in leukocyte rolling velocity and increases in numbers of adherent leukocytes, increased numbers of leukocytes had emigrated into surrounding tissues of Fps-null mice at all time points examined (Figure 3-2B; P=0.005).

We also examined hemodynamic parameters in the cremaster muscle venules of LPS-challenged mice [172]. There were no differences in RBC velocity between genotypes. However, a lower vessel wall shear rate was observed in LPS-challenged Fps-null mice (Table 3-1; P=0.021) which was consistent with the increased number of adherent cells observed in Fps-null mice.

3.3.2 Leukocytes from Fps-null mice displayed increased rates of transmigration in response to a local LPS challenge.

Having observed an increase in both the number of leukocytes adhered to the vessel wall, and the number of extravasated leukocytes in Fps-null mice, we next sought to determine if there might be a difference in the rate at which leukocytes were undergoing diapedesis in wild-type and Fps-null mice. RLOT analysis revealed that leukocytes transmigrated the vessels in Fps-null mice over 30% faster than in wild-type animals (Figure 3-3; 8.0±1.1 min vs. 11.8±0.7 min. P=0.032). Together with the parameters examined above, this confirmed that mice lacking Fps are more sensitive to inflammation induced by the localized injection of LPS.

3.3.3 Fps regulates neutrophil migration into the peritoneal cavity in response to thioglycollate.

Having observed an increase in the number of extravasated leukocytes in the tissues
Figure 3-1: Defects in leukocyte rolling in the cremaster venules of Fps-null mice challenged with LPS. Mice were injected intrascrotally with 0.05 µg/kg LPS. The contralateral cremaster muscle was externalized for observation, and the indicated parameters were assessed by intra-vital video microscopy. Number of rolling cells per vessel was calculated by dividing rolling flux by rolling velocity, and multiplying by vessel length (100 µm). P values were obtained by two-way ANOVA for repeated measures, and are a comparison of wild type vs. fps^-/. For all time points, n=4 or 5.
Figure 3-2: Enhanced leukocyte adherence and emigration in the cremaster venules of Fps-null mice challenged with LPS. Mice were injected intrascrotally with 0.05 µg/kg LPS. The contralateral cremaster muscle was externalized for observation, and the indicated parameters were assessed by intra-vital video microscopy. P values were obtained by two-way ANOVA for repeated measures, and are a comparison of wild type vs. fps−/−. For all time points, n=4 or 5.
Table 3-1: Hemodynamic parameters in cremaster post-capillary venules of wild type and Fps-null mice challenged with LPS. Mice were injected intrascrotally with 0.05 µg/kg LPS. The contralateral cremaster muscle was externalized for observation, and the indicated parameters were assessed by intra-vital video microscopy. Vessel diameter and RBC velocity were measured, and shear was calculated as described in materials and methods. **P=0.021 for wall shear rate; *P=0.066 for RBC velocity. P values were obtained by two-way ANOVA for repeated measures, and are a comparison of wild type vs. fps−/−. For all time points, n=5.

<table>
<thead>
<tr>
<th>Geonotype</th>
<th>Treatment</th>
<th>Vessel Diameter (µm)</th>
<th>*RBC Velocity (mm/sec)</th>
<th>**WS Rate (sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>3 hrs. post LPS</td>
<td>26.9 ± 2.0</td>
<td>1.08 ± 0.05</td>
<td>250 ± 40</td>
</tr>
<tr>
<td>Wild Type</td>
<td>3.5 hrs. post LPS</td>
<td>28.6 ± 2.1</td>
<td>1.48 ± 0.30</td>
<td>255 ± 40</td>
</tr>
<tr>
<td>Wild Type</td>
<td>4 hrs. post LPS</td>
<td>28.6 ± 2.1</td>
<td>1.30 ± 0.17</td>
<td>197 ± 11</td>
</tr>
<tr>
<td>Fps-null</td>
<td>3 hrs. post LPS</td>
<td>30.0 ± 2.8</td>
<td>0.98 ± 0.07</td>
<td>164 ± 4</td>
</tr>
<tr>
<td>Fps-null</td>
<td>3.5 hrs. post LPS</td>
<td>28.3 ± 2.9</td>
<td>1.08 ± 0.10</td>
<td>177 ± 8</td>
</tr>
<tr>
<td>Fps-null</td>
<td>4 hrs. post LPS</td>
<td>28.8 ± 2.7</td>
<td>1.06 ± 0.04</td>
<td>200 ± 4</td>
</tr>
</tbody>
</table>
Figure 3-3: Fps regulates time required for leukocyte diapedesis in response to local challenge with LPS. RLOT was used to assess transmigration of leukocytes by intra-vital video microscopy. Diapedesis time was recorded as the time required for individual leukocytes to escape from vessel into surrounding tissue after initial adherence. Data for each mouse is shown in separate columns. Mean for each mouse is represented by a solid line, and mean for each genotype is represented by a broken line. Student’s t-test P<0.0001, wild type vs. fps⁻⁻. 
surrounding the cremaster muscle in Fps-null mice, we next asked if this result could be replicated in the peritoneal cavity. To address this, mice were injected IP with thioglycollate to induce a peritoneal inflammation, and we then examined the number and composition of cells recovered by peritoneal lavage. As expected, there was an increase in the total number of cells recruited to the peritoneal cavities of Fps-null mice (Figure 3-4A). Flow cytometric analysis of these peritoneal cells using lineage specific surface markers revealed a significant increase in the percentage of Ly6G/CD11b double positive neutrophils recovered from the peritoneum of Fps-null mice (Figure 3-4B; P=0.025). We also analysed the percentage of Ly6G–ve/CD11b +ve cells recruited to the peritoneal cavity, but no difference was found between wild type and Fps-null mice (Figure 3-4B).

3.3.4 Fps regulates LPS-induced changes in PSGL-1 and CD11b surface expression on peripheral blood neutrophils.

P-selectin glycoprotein ligand-1 (PSGL-1) is thought to be one of the major leukocyte adhesion molecules responsible for the initiation of rolling along the activated endothelium, through its binding to P-selectin, and to a lesser extent E-selectin [167]. The integrin CD11b is known to participate in inflammation-induced leukocyte adherence to vessel endothelium. Since we observed aberrant behaviour of Fps-null leukocytes with respect to both rolling and adherence, we examined the expression of both of these molecules on peripheral blood neutrophils (PBNs) from wild type and Fps-null mice stimulated ex vivo with LPS. In PBNs from Fps-null mice, there was an increased retention of PSGL-1 surface expression after LPS stimulation, as compared to those isolated from wild type mice (Figure 3-5A; P=0.023). In the same experiment we also assessed the levels of CD11b surface expression subsequent to LPS stimulation. Surprisingly, we observed a trend toward reduced LPS-induced CD11b up-regulation on fps/− neutrophils, although this difference did not reach statistical significance (Figure 3-5B; P=0.086). An analysis of PSGL-1 and CD11b expression subsequent to LPS stimulation on monocytes was
Figure 3-4: Effect of Fps on neutrophil migration into the peritoneal cavity in response to thioglycollate. Mice were challenged I.P. with 1 mL of 4% thioglycollate, and 4 hours later were euthanized and peritoneal lavage performed. A: Total cell number recovered by lavage. P value was obtained by Student’s t-test. n=5. B: Assessment of Ly6G and CD11b expression of recruited cells as measured by flow cytometry. n=3. Ly6G/CD11b +ve P=0.025; Ly6G –ve/ CD11b+ve P=0.574 by Student’s t-test.
Figure 3-5: Effect of Fps on PSGL-1 and CD11b expression on peripheral blood neutrophils stimulated with LPS. Peripheral blood was collected by cardiac puncture, and stimulated with LPS at 100 ng/mL. At the indicated times, an aliquot of blood was removed and placed in TBS-V on ice. Expression of PSGL-1 and CD11b was then measured by flow cytometry. A: and B: neutrophils; C: and D: monocytes. A: and C: Levels of surface expression of PSGL-1. B: and D: Levels of surface expression of CD11b. For all time points, n=3. For all plots, P values were obtained by two-way ANOVA for repeated measures, and are a comparison of wild type vs. fps−/−.
also performed. As with neutrophils, monocytes showed a decrease in the level of PSGL-1 surface expression, with a corresponding increase in CD11b levels; however, no difference between wild type and Fps-null cells was observed (Figure 3-5C and D).

3.4 Discussion

Using mice targeted with a loss-of-function mutation in fer, previous studies have established a role for Fps-related Fer kinase in the physiological response of leukocytes to challenge with LPS [95]. We have also previously implicated Fps in the regulation of inflammation by showing that Fps-null mice are more susceptible to systemic challenge with LPS [67], and that this defect is likely due to an over production of TNF-α by fps-/- macrophages [170]. Here we provide further in vivo evidence for the role of Fps in regulating the physiological response to LPS.

In the innate immune response to LPS, there is an ordered series of leukocyte-endothelial cell interactions which allow leukocytes to exit the vessel lumen, and migrate towards sites of injury or inflammation. This process in one of the hallmarks of inflammation, and is thought to be responsible for much of the resulting tissue damage. Here we show that during every step of this process (leukocyte rolling, adherence, transmigration time, and number of extravasated cells), there is a defect in Fps-null mice relative to wild type counterparts which is consistent with increased inflammation (Figures 3-1 and 3-2). In Figure 3-1C, we observed that there was a significant decrease in the number of rolling cells in wild type vessels between 3 and 3.5 hours post challenge, while in Fps-null vessels this decrease was not apparent until 4 hours post challenge, which may indicate a defect or a delay in the resolution of inflammation in Fps-null animals. Overall, these defects correspond to a situation in which mice lacking the Fps kinase experience a heightened state of inflammation when challenged locally with LPS. This increase in inflammation in the Fps-null mice is most likely detrimental to the animal, which is consistent with the higher mortality rates observed in Fps-null mice challenged systemically with LPS.
compared to their wild type cohorts [67].

The increase in thioglycollate-induced recruitment of neutrophils to the peritoneal cavity of Fps-null mice (Figure 3-4) is in agreement with the increased leukocyte recruitment to the inflamed cremaster muscle (Figure 3-2B). A simple explanation for this difference might be if there were differences in the basal levels of neutrophils between wild type and fps\(^{-/-}\) mice. However, previous work with fps\(^{-/-}\) mice has shown that there is no difference in peripheral blood levels compared to wild type in unchallenged mice (S.P. and P.G. unpublished results). With this in mind, the decrease in LPS-induced up-regulation of CD11b in fps\(^{-/-}\) neutrophils is particularly interesting since previous studies have shown that mice deficient for CD11b display increased thioglycollate-induced recruitment of neutrophils into the peritoneal cavity, and that this is due to a defect in apoptosis in the CD11b deficient mice [169]. This led us to initially speculate that the increased recovery of peritoneal neutrophils from thioglycollate challenged fps\(^{-/-}\) mice (Figure 3-4B) might correlate with a defect in apoptosis due to their decreased ability to up-regulate CD11b (Figure 3-5B, and see below). However, experiments examining \textit{in vivo} neutrophil apoptosis subsequent to thioglycollate challenge revealed no difference at 4 hours post challenge. At 24 hours post challenge, there was a modest decrease in the percentage of apoptotic Fps-null neutrophils relative to wild type, but this difference did not reach statistical significance (24.9 ± 1.7 wt vs. 18.5 ± 0.1 fps\(^{-/-}\); P=0.061, data not shown).

Differences in the levels of surface expression of PSGL-1 were also observed on Fps-null neutrophils, subsequent to LPS challenge. PSGL-1 is known to be critically important in mediating the rolling of neutrophils along inflamed endothelium [168]. Therefore, our observation that there was increased retention of PSGL-1 on the surface of Fps-null neutrophils subsequent to LPS challenge, might at least partially account for the decreases in leukocyte rolling velocity, and the increased number of rolling leukocytes in Fps-null mice \textit{in vivo} (Figure 3-1B,C). We have previously shown a role for Fps in the regulation of TLR4 and transferrin receptor endocytosis, as well as bacterial phagocytosis, suggesting a more general function for
Fps in cytoskeletal reorganization associated with endocytosis or phagocytosis [170]. Surface distribution of PSGL-1 appears to involve a tyrosine kinase-dependent cytoskeletal reorganization process [173] and surface receptor shedding [174]. Likewise, CD11b has been shown to interact with the cytoskeleton (reviewed in [175]), changes in which have been proposed to regulate both the mobility of the integrin within the membrane, as well as its binding activity[176]. Therefore, our observations with respect to PSGL-1 and CD11b lend further support to a more generalized function of Fps in cytoskeletal regulation.

The observed differences in PSGL-1 expression are statistically significant; however, the magnitude of the differences are not overly large, with the total area under the curve for Fps-null cells being only 9% greater than for wild type cells. Therefore, although we have established a role for Fps in the regulation of PSGL-1 surface expression, and this is likely to have an effect on leukocyte migration to sites of inflammation, we must also consider the possibility of a role for Fps in other cell types, and in the regulation of other adhesion molecules (see discussion below). CD11b has been shown to be a key modulator of neutrophil adherence [169]. We observed increases in leukocyte adherence in Fps-null mice in vivo, yet surprisingly, there was less CD11b up-regulation on Fps-null neutrophils compared to wild type after in vitro LPS challenge. There are several possible explanations for this discrepancy. First, in the cremaster inflammation model used in this study, leukocyte rolling on the endothelium is thought to be a necessary precursor to leukocyte adherence, and slower leukocyte rolling velocities promote firm adhesion [177]. Therefore, the observed increase in the number of rolling leukocytes and the decrease in leukocyte rolling velocity in Fps-null mice (Figure 3-1B,C), might account for the observed increase in leukocyte adherence. Second, as shown in Figure 3-5B, although CD11b surface expression was not up-regulated in Fps-null neutrophils to the same extent as it was in wild type cells, it was still up-regulated by over 300% of control levels by four hours post LPS stimulation (Figure 3-5B, fps-/control MFI = 49.6, MFI at 4 hours post stimulation =152.1). Therefore, the level of up-regulation of CD11b in Fps-null neutrophils was likely sufficient to
achieve firm adhesion, and the increased number of adherent leukocytes in Fps-null animals in vivo might be due to the increased number of rolling cells in these animals. Lastly, in order to facilitate proper adhesion and extravasation, CD11b must change from a low to a high affinity status [178]. Therefore, it is possible that even though there was less CD11b expressed on the surface of Fps-null cells, a greater proportion of it might have been in the high affinity state relative to wild type cells, thereby preferentially promoting the adhesion of Fps-null cells.

In addition to PSGL-1 and CD11b examined here, various other adhesion molecules are expressed on neutrophils and known to play a role in leukocyte adhesion. For instance, a recent article by Hidalgo et. al. sought to elucidate the complete spectrum of E-selectin binding partners expressed on neutrophils. Their results demonstrate roles for PSGL-1 in the initiation of rolling, but also for E-selectin ligand-1 (ESL-1) in the stabilization and transition to steady rolling, and for CD44 in the control of leukocyte rolling velocities[115]. Interestingly, the authors go on to show that all three E-selectin ligands have a role in regulating neutrophil recruitment in a thioglycollate-induced peritoneal inflammation model[115]. With this in mind, it would be interesting to investigate the possibility that Fps is playing a role in the regulation of other neutrophil adhesion molecules, in addition to PSGL-1 and CD11b.

Gene-targeted Fer-deficient mice [88], also displayed defects in leukocyte behavior very similar to those observed in the present study [95]. Since Fps and Fer have very similar structures, and might therefore regulate some of the same processes, it is intriguing to speculate that a double fps-fer knockout [94] will display an even more severe phenotype than either of the single knockouts. These studies are currently underway.

In addition to leukocyte activation, activation of the vascular endothelium is an essential step in promoting leukocyte rolling, adhesion, and extravasation. Fps and Fer are both expressed in vascular endothelial cells as well as in leukocytes; therefore, it will be important to determine how much of the observed hyperinflammatory phenotype seen in either Fps- or Fer-deficient mice is due to loss of Fps or Fer expression in the endothelium versus in the leukocytes. In support of
this idea, a recent report shows a role for cortactin phosphorylation in modulating E-selectin and ICAM-1 clustering and actin remodeling in [170] endothelial cells, thereby affecting polymorphonuclear cell (PMN) transmigration in vitro [179]. In that study, Src family kinases were implicated in cortactin phosphorylation. However, other studies have implicated Fer in phosphorylation of cortactin [86, 88, 89]. Fer activity was also shown to be required for cortactin phosphorylation and strengthening of N-cadherin based cell-cell interactions [180]. Furthermore, preliminary results suggest that Fps might also participate in the phosphorylation of HS-1 (S.A.P. and P.A.G., unpublished observations), which is a cortactin paralog expressed in hematopoietic cells. Therefore, the possibility exists that Fps may have a role in regulating leukocyte recruitment through activity in endothelial cells, as well as in leukocytes.

Finally, there is one study implicating Fer in the regulation of another important endothelial adhesion molecule, platelet/endothelial cell adhesion molecule (PECAM)-1 [146]; and interestingly, PECAM-1 knockout mice have also been shown to have a hyperinflammatory response to LPS[181]. Additional studies will be required to further elucidate the potential roles of Fps and Fer kinases in regulating the cell-cell and cell-matrix receptor systems which control transendothelial migration of immune cells and cancer cells.

In summary, we show that mice lacking Fps have an increased response to LPS induced inflammation, with defects in leukocyte rolling, adhesion and extravasation. We go on to show that these defects might be due to a role for Fps in regulating the surface expression of the adhesion molecule PSGL-1 on the surface of neutrophils. Further investigation into the role of Fps in regulating receptor expression on the surface of endothelial cells is required to elucidate the precise molecular role of this cytoplasmic tyrosine kinase in the inflammatory response.
Chapter 4

The Fps/Fes kinase regulates TNF-α-induced cell death in macrophages.

4.1 Introduction

The fps/fes gene (hereafter referred to as fps), encodes a 92 kDa Fps protein, belonging to subgroup IV of the non-receptor protein tyrosine kinases[43, 84]. The closely related Fer protein is the only other member of this subgroup of kinases[42, 84, 85]. The expression of Fps has been reported in hematopoietic cells including monocytes/macrophages, neutrophils, mast cells, platelets, and erythrocytes; as well as in some neuronal, epithelial and vascular endothelial cells. In contrast, during development Fps is expressed in all three germ layers [46, 48, 133] (reviewed in [36]).

The Fps and Fer kinases consist of a C-terminal kinase domain, a central SH2 domain and an N-terminal Fps/Fer/CIP4 homology (FCH) domain associated with three coiled-coiled domains [36]. The FCH domain it is thought to mediate associations with phospholipid components of membranes as well as the cytoskeleton [15, 16, 22], and is unique to Fps and Fer among protein-tyrosine kinases.

When the body encounters a foreign substance, an inflammatory response is elicited, and three key processes serve to regulate this process. First, there is an inflammatory stimulus, resulting in the activation of signalling pathways in cells such as macrophages, leading to the secretion of pro-inflammatory molecules at the site of injury. Secondly, the release of these molecules leads to changes in both the vasculature, as well as circulating blood cells, resulting in the recruitment of inflammatory cells to the site of injury. Lastly, apoptosis of these recruited cells serves to dampen inflammation by reducing their life span, thereby limiting the amount of pro-inflammatory molecules released.

Fps is expressed very highly in macrophages (while Fer is expressed to a lesser extent),
and high levels of expression of both of these kinases is also found in the vascular endothelium. Due to the importance of these two groups of cells in inflammation, we have explored the possibility of a role for these tyrosine kinases in regulation of the innate immune response.

We have previously reported increased mortality in Fps knockout (fps−/−) mice in response to LPS challenge in vivo [67], which was at least partially due to increased in vivo levels of TNFα caused by enhanced NF-κB signalling in macrophages [170]. Mice deficient in the Fer kinase also displayed increased LPS-induced neutrophil adhesion to venules and extravasation in the cremaster muscle [95] and the small intestine submucosa [96]. Furthermore, we have shown a role for Fps in regulating leukocyte rolling, adhesion, extravasation and recruitment subsequent to LPS challenge, possibly due a prolonged retention of surface PSGL-1 on neutrophils from Fps-null mice subsequent to stimulation with LPS[182].

Here we provide evidence of a role for Fps in cell death in macrophages in response to inflammatory stimuli. Upon LPS challenge, we observe an increased retention of macrophages in the peritoneal cavity of mice lacking Fps. We go on to show that this is likely due to a defect in TNF-α-induced death in Fps-null macrophages. Furthermore, in the absence of Fps, we observed an increase in NF-κB signalling, and a decrease in poly (ADP ribose) polymerase (PARP) induction, both of which correlate with increased cell survival. Finally, we show a possible role for Fps in the resolution of inflammation.

4.2 Materials and Methods

4.2.1 Intra-peritoneal LPS challenge and peritoneal lavage

Mice were injected intra-peritoneally with 100 μl PBS containing 1 mg/kg LPS or PBS alone (Figure 4-1), or 1.5 mL of 4% thioglycollate broth (Figure 4-5), using a 1 mL syringe fitted with a 5 gauge needle. For Figures 4-2, 4-3, and 4-4, lavage was performed on unchallenged mice. Mice were sacrificed by chloroform inhalation, and peritoneal lavage was performed twice
by injecting 5 mL of lavage media [RPMI 1640 (Gibco) with 10 mM HEPES, 10 U/mL heparin (Sigma), 5 mM EDTA, 1% (vol/vol) antibiotic-antimycotic (aa) (Gibco), 50 µM α-monothioglycerol] using a 5mL syringe and 23 gauge needle. Cells were extracted, pelleted at 700RPM for five minutes, re-suspended in 5 mL pre-warmed culture media [RPMI 1640 (Gibco) with 5% (vol/vol) FBS (Hyclone), 50 µM α-monothioglycerol, 10 mM HEPES, 2 mM glutamine (Gibco) 1% aa. (Gibco)] and counted.

4.2.2 Stimulation of peritoneal macrophages.

After lavage, cells were diluted to a concentration of 5.0 x 10^5 cells/mL, and 1 mL aliquots placed in 12 well culture plates and incubated for 2 hours at 37°C. Cells were then stimulated with 10 ng/mL TNF-α in 0.5 mL culture media for the indicated times at 37°C. To stop stimulations, media was aspirated and 1 mL cold TBSV [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 100 µM vanadate] was added to each well and plates placed on ice. Soluble cell lysates were prepared by aspirating TBSV and scraping cells into 150 µL of 2xSDS sample buffer. Lysates were either frozen at -20°C, or resolved on 7.5% or 11% SDS-polyacrylamide gels. Proteins were transferred by semi-dry blotting to Immobilon-P membrane (Millipore), blocked with either 5% milk powder or 5% BSA in TBS-Tween and probed with the following primary antibodies: Rabbit α-Fps/Fer [48], mouse α-pY99 (200 µg/mL, Santa Cruz Biotechnology, Santa Cruz, CA), goat α-actin (200 µg/mL, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit α-PARP (Cell Signalling Technology, Danvers, MA), rabbit α-phospho-p65-NF-κB (Cell Signalling Technology, Danvers, MA), rabbit α-IκBα (Cell Signalling Technology, Danvers, MA).

4.2.3 Apoptosis analysis

For Figure 4-3, cells were collected by lavage as described above, and incubated for 2 hours at 37°C in culture media in 4 mL polystyrene snap cap tubes. Tubes were used for cell culture to prevent macrophages from adhering to the surface, thus preventing any membrane
disruption (and false positive annexin V staining) associated with scraping cells off of a plate. Cells were then stimulated with 10 ng/mL TNF-α in 0.5 mL culture media for the indicated times, and stimulations stopped by adding 3 mL of ice-cold annexin V binding buffer [2.5 mM CaCl₂, 140 mM NaCl, 10 mM Hepes/NaOH, pH 7.4]. Cells were spun, and re-suspended in 100 µL of annexin V binding buffer containing 1 µl of FITC-conjugated annexin V (BioVision, Mountain view, CA), 2.5 mg/mL propidium iodide (Sigma), and 1 µg/mL phycoerythrin-conjugated anti-mouse F4/80 (Caltag Laboratories, Burlingame, CA), for 15 minutes at room temperature in the dark. A further 400 µL of ice-cold annexin V binding buffer was added, and cells were placed on ice and analysed by flow cytometry. For Figure 4-2, cells were collected by lavage, cultured in 12 well plates, and stimulated with TNF-α as described above. Following stimulation, cells were removed from the plate by adding 2 mL of a pre-warmed 1:1 solution of culture media and 10 mM for 20 minutes at 37°C. Cells were then gently pipetted up and down to loosen adherent cells, pelleted, and fixed by adding 3 mL of cold 70% ethanol while vortexing. Methanol was aspirated and cells were washed and resuspended in 3 mL PBS, containing 3.3 µg/mL RNase A, and 3.3 µg/mL propidium iodide. Cells were vortexed, and cell death analysis performed by flow cytometry.

4.2.4 Cell recruitment analysis.

After counting, cells collected by lavage 24 hours after LPS challenge (Figure 4-1) or 5 days after thioglycollate challenge (Figure 4-5), were washed once and resuspended in ice-cold PAB (PBS containing 0.3% BSA [w/v], 0.1% sodium azide [w/v]). 1 x 10⁶ cells were then transferred to 5 mL snap cap tubes, and incubated with 1 µg/mL PE-conjugated anti-mouse F4/80 (Caltag Laboratories, Burlingame, CA), 1.0 µg/mL PE-conjugated anti-mouse Ly6G, and 0.5 µg/mL FITC-conjugated anti-mouse CD11b (BD Biosciences), for 15 minutes at room temperature in the dark. Cells were washed once with 4 mL of ice-cold PAB, and analyzed by
flow cytometry.

4.3 Results

4.3.1 Fps promotes disappearance of macrophages from the peritoneal cavity of mice challenged with LPS.

We have previously shown that Fps-null mice display an increased recruitment of neutrophils in response to an inflammatory challenge, both to the cremaster muscle in a locally induced LPS challenge, and to the peritoneal cavity in response to thioglycollate[182]. Furthermore, we have identified Fps as being important in a model of intra-peritoneal challenge with LPS[67], and that the role of Fps in this model was to regulate the secretion of cytokines from macrophages[170]. We therefore sought to examine whether or not there may be differences in the recruitment of inflammatory cells to the peritoneal cavity, subsequent to challenge with LPS. We observed that twenty-four hours after LPS injection, there was a 36% decrease in the number of cells recovered from the peritoneal cavity of wild type mice, as compared to control levels ($3.49 \times 10^6$ vs. $2.56 \times 10^6$, $P=0.007$, Figure 4-1A), while the number of cells recovered from Fps-null mice remained constant between challenged and unchallenged animals ($3.68 \times 10^6$ vs. $3.79 \times 10^6$, Figure 4-1A).

Having observed a retention of cells in the peritoneal cavity of LPS-challenged Fps-null mice, we next sought to assess which cell type(s) was/were responsible for these differences. We used flow cytometry to analyse the expression of macrophage (F4/80) and neutrophil (Ly6G) markers on cells recovered by peritoneal lavage both before and after LPS challenge. Our results show that upon LPS challenge of wild type mice, there is a 23% drop in the macrophage fraction in cells recovered from the peritoneal cavity ($70.5\%$ vs. $47.4\%$, $P=0.058$, Figure 4-1B).
Figure 4-1: Fps regulates disappearance of macrophages from peritoneal cavity subsequent to LPS challenge. Wild type and Fps-null mice were injected with 1 mg/kg of LPS into the peritoneal cavity, and peritoneal lavage was performed 24 hours later. Cells were counted and flow cytometry performed. A: Total number of cells recovered by peritoneal lavage. B: Flow cytometry of cells recovered by lavage using phycoerythrin-labelled F4/80 (macrophage marker) and FITC-labelled CD11b. For squares marked in red, P=0.058 control vs. LPS stimulated by Student’s t-test. C: Flow cytometry of cells recovered by lavage using phycoerythrin-labelled Ly6G (neutrophil marker) and FITC-labelled CD11b. For squares marked in blue, P<0.014, control vs. LPS stimulated by Student’s t-test. For all conditions, n=2 to 5.
Expressed in a different way, this corresponds to a decrease of 52% (from $2.46 \times 10^6$ to $1.19 \times 10^6$) in the actual number of macrophages recovered from the peritoneal cavity. This decrease in macrophage recovery after LPS injection was not observed in Fps-null animals (Figure 4-1B). We also observed a substantial LPS-induced recruitment of neutrophils to the peritoneal cavity; however, there was no difference between genotypes (Figure 4-1C).

4.3.2 Fps promotes cell death in macrophages stimulated with LPS and TNF-α.

The observed differences in the numbers of macrophages retained in the peritoneal cavities of Fps-null and wild type mice led us to investigate the possibility that there might be a role for Fps in regulating cell death in response to inflammatory stimuli. This was assessed by using flow cytometry to quantitate the appearance of a sub-G1 peak in fixed cells stained with propidium iodide (PI). When stimulated for 6, 12, and 24 hours with LPS, cells lacking Fps displayed reductions in apoptosis relative to wild type cells of 11%, 14%, and 22%, respectively (Figure 4-2A). However, since it has been suggested that LPS-induced cell death in cultured macrophages is caused by the autocrine action of TNF-α[183], we decided to examine the possibility that stimulation with this cytokine would also result in a difference in the level of cell death between wild type and Fps-null cells. Indeed, apoptosis in TNF-α treated Fps-null macrophages was 24%, 39%, and 43% less than in wild type macrophages at 6, 12, and 24 hours respectively (Figure 4-2B).

4.3.3 The reduction in cell death in TNF-α-stimulated Fps-null macrophages is due to decreases in both apoptosis and necrosis.

Cell death analysis using PI staining to measure nuclear condensation is reasonably specific for identifying apoptotic cells; however, a small percentage of cells undergoing necrosis will also display nuclear condensation, such that they gate into a sub-G1 peak. Therefore, we
Figure 4-2: Relative cell death in \textit{fps-null} and wild type peritoneal macrophages stimulated with LPS and TNF-\textit{c}.

Cells were collected by peritoneal lavage from unchallenged mice, and stimulated with 10 ng/mL TNF-\textit{c}, or 100 \text{g/mL} LPS for 0, 6, 12, and 24 hours. Cells were then fixed, and cell death analysis performed by flow cytometry. A: Cell death induced by stimulation with LPS. B: Cell death induced by stimulation with TNF-\textit{c}. P values were obtained using Student’s t-test. For all time points, \( n = 4 \) (LPS stimulation) or 5 (TNF-\textit{c} stimulation).
sought to define more specifically what percentage of cell death observed previously were attributable to apoptosis versus necrosis. For this analysis we chose to stimulate cells with only TNF-α for two reasons: first, the magnitude of the difference observed in cell death between wild type and Fps-null cells was much greater for stimulations with TNF-α as compared to stimulations with LPS (Figure 4-2A and B). Secondly, it has previously been shown that most of the apoptosis in macrophage cultures stimulated with LPS can be contributed to the resulting secretion and autocrine function of TNF-α[183]. Using flow cytometry, we observed decreases in the fraction of apoptotic (annexin V +ve/PI -ve) Fps-null macrophages at 6, 12, and 24 hours post stimulation, as compared to wild type (Figure 4-3A). We next assessed the level of necrosis (annexin V +ve/PI +ve) in TNF-α-stimulated macrophages, and again observed decreases in Fps-null cells at 6, 12, and 24 hours post stimulation (Figure 4-3B). Interestingly, the magnitude of the differences between wild type and Fps-null cells was much greater for necrotic versus apoptotic macrophages (Figure 4-3A and B).

4.3.4 Fps regulates degradation of IκB-α, phosphorylation of NF-κB and induction of PARP in macrophages stimulated with TNF-α.

In Figure 4-3A and B, we showed differences in the level of both apoptosis and necrosis in Fps-null macrophages stimulated with TNF-α, as compared to wild type. In an attempt to elucidate how Fps was participating in these processes, we again stimulated macrophages with TNF-α, and used western blotting to examine some of the key signalling pathways known to regulate cell survival. Relative to wild type macrophages, Fps-null macrophages displayed increased activation of the pro-survival NF-κB pathway. Specifically, there was increased degradation of IκB-α at 15 and 30 minutes post TNF-α stimulation in Fps-null macrophages, with a corresponding increase in the phosphorylation of p65NF-κB at 15, 30, 60 and 120 minutes post stimulation (Figure 4-4). We also examined the levels of PARP, a protein known to be cleaved
Figure 4-3: Fps regulates apoptosis and necrosis in response to TNF-α. Cells were collected by peritoneal lavage from unchallenged mice, and stimulated with 10 ng/mL TNF-α, for 0, 6, 12, and 24 hours, and cell death was then analyzed by flow cytometry. A: Percentage of apoptotic cells as measured by annexin V staining. B: Percentage of necrotic cells as measured by double positive annexin V and propidium iodide staining. P values were obtained by using Student’s t-test. For all time points, n = 5.
by caspases in response to many apoptotic stimuli[184, 185], a process which serves as a general marker of apoptosis. At the times examined, there did not appear to be any difference in the amount of cleaved PARP in wild type or fps⁻/⁻ macrophages; and indeed there was very little TNF-α-induced PARP cleavage in either wild type or Fps-null macrophages (Figure 4-4). However, while TNF-α induced a significant up-regulation of full length PARP in wild type macrophages, this was not apparent in Fps-null macrophages (Figure 4-4).

4.3.5 Fps promotes resolution of thioglycollate-induced inflammation.

We have previously observed an increased thioglycollate-induced recruitment of neutrophils to the peritoneal cavity of mice lacking Fps[182]. Since we observed differences in cell death in macrophages in response to inflammatory stimuli (Figures 4-2 and 4-3), and it is known that apoptosis can promote the resolution of inflammation, we examined whether or not there might be a role for Fps in resolution of inflammation. Wild type and Fps-null mice were injected i.p. with thioglycollate, and five days later peritoneal cellularity was assessed by peritoneal lavage followed by flow cytometry analysis. This revealed a 2 fold increase in the number of cells (5.8 x10⁶ wild type vs. 2.5 x10⁶ fps⁻/⁻, Figure 4-5), recruited to the peritoneal cavity of wild type mice, as compared to Fps-null counterparts. Of these recruited cells, a greater proportion were macrophages in wild type mice, as compared to Fps-null mice (Figure 4-5, inset). The role of these “late” macrophages is to clear away previously recruited inflammatory neutrophils which have undergone apoptosis, thereby helping to resolve inflammation. With this in mind, we also analyzed what proportion of the cells recovered by lavage were either macrophages or neutrophils. Flow cytometry analysis revealed that 5 days post thioglycollate challenge, there was a 2.5 fold increase in the percentage of neutrophils remaining in the peritoneal cavities of Fps-null mice, compared to wild type counterparts (Figure 4-5, inset).
Figure 4-4: Absence of Fps correlates with increased NF-κB activation and decreased induction of PARP in macrophages stimulated with TNF-α. Cells were collected by peritoneal lavage from unchallenged mice, and stimulated with 10 ng/mL TNF-α, for the indicated times. Soluble cell lysates were prepared, resolved on SDS-PAGE gels, transferred to membranes, and probed with the indicated antibodies.
**Figure 4-5: Fps promotes resolution of thioglycollate-induced inflammation.** Wild type and Fps-null mice were injected with 1 mL of 4% thioglycollate broth into the peritoneal cavity. Peritoneal lavage was performed 5 days later, and total number of cells recovered was recorded. Inset: Flow cytometry analysis of cells recovered by lavage using phycoerythrin-labelled F4/80 (macrophage marker), and phycoerythrin-labelled Ly6G (neutrophil marker). P values were obtained using the Student’s t-test.
4.4 Discussion

Previous studies have provided a basis for Fps as an important player in modulating the response to LPS-induced inflammation[67], both through its ability to modulate the secretion of pro-inflammatory cytokines[170], as well as its role in controlling the number of leukocytes homing to areas of inflammation [182]. Here, we report the participation of Fps in another key step in the regulation of inflammation, the apoptosis of immune cells.

Much of the work to date examining the role of apoptosis in resolution of inflammation has focused on the fate of neutrophils, subsequent to their recruitment to sites of inflammation[118, 121]. These studies clearly show that neutrophil apoptosis is an essential process in the resolution of inflammation, for two reasons. First, apoptosis of neutrophils promotes their phagocytic clearance from sites of inflammation by macrophages, a process which protects the surrounding tissue from the pro-inflammatory contents inside the neutrophil[121, 122]. Secondly, phagocytosis of apoptotic neutrophils promotes the release of anti-inflammatory mediators such as IL-10[120].

Here, we report evidence suggesting that Fps plays a role in the recruitment of macrophages to the inflamed peritoneal cavity. Further analysis implicates Fps in the regulation of macrophage cell death, both apoptotic and necrotic, subsequent to TNF-α stimulation. Our results also showed that in the absence of Fps, TNF-α-induced NF-κB activation was increased, while PARP expression was not induced. Finally, we suggest that the decrease in apoptosis in Fps-null macrophages leads to a failure to properly resolve inflammation.

In Figure 4-1, we showed that the 28% decrease in the number of cells recovered from the peritoneal cavity of wild type mice after LPS challenge was attributable to a decrease in the number of macrophages recovered; interestingly, there was not a corresponding decrease in peritoneal macrophages in Fps-null mice (Figure 4-1A and B). We go on to show decreases in Fps-null macrophage apoptosis and necrosis in vitro (Figures 4-2, 4-3), therefore suggesting that these differences in cell death were responsible for the observed in vivo differences in LPS-
induced changes in peritoneal macrophage cellularity Figure 4-1. However, others have suggested that the disappearance of macrophages from the inflamed peritoneum is not due to apoptosis, but due to their migration to draining lymph nodes[186]. In a subsequent study, these same authors reported that this clearance of macrophages from the peritoneum during the resolution of inflammation is controlled, at least partially by the β1 integrins very late antigen (VLA)-4 and VLA-5[187]. Their study proves this as the principal method of clearance of thioglycollate-elicited inflammatory macrophages from the peritoneal cavity subsequent to inflammation; however, the idea that resident peritoneal macrophages are also cleared in this manner subsequent to inflammation is not convincingly shown, and apoptosis as a method of their disappearance was not disproved[187].

Activation of NF-κB is known to induce secretion of pro-inflammatory cytokines[138], but it has also been shown to promote cell survival[188]. We have previously shown an increase in NF-κB activation downstream of LPS stimulation in macrophages lacking Fps[170], and here we show for the first time an increase in NF-κB activation downstream of TNF-α stimulation as well (Figure 4-4). Therefore, it was not surprising that we observed increased survival of Fps-null macrophages that were stimulated with both LPS and TNF-α. In LPS-stimulated macrophages, Fps was shown to promote the endocytosis of the LPS receptor TLR-4[170], a process known to down-regulate NF-κB signalling[139]. With this in mind, it will be interesting to investigate a possible role for Fps in endocytosis of TNF receptor 1 (TNFR1), especially since internalization of this receptor is necessary for a switch from pro-inflammatory NF-κB signalling, to pro-apoptotic caspase activation[189].

In contrast to an increase in NF-kB activation in response to TNF-α stimulation in Fps-null macrophages, we observed a complete failure to up-regulate the nuclear enzyme PARP (Figure 4-4). Cleavage of PARP is an early event in apoptosis[190], and is often used as a marker for cells undergoing this process[191]. However, it has been observed that activation of PARP
can cause necrotic cell death[192, 193], presumably due to depletion of cellular energy reserves[194]. Indeed, our results seem to agree with this role for PARP, since Fps-null cells that did not up-regulate PARP upon TNF-α stimulation (Figure 4-4), were also resistant to necrotic cell death (Figure 4-3B).

Neutrophils can be recruited to an inflamed peritoneum in as little as 2 hours. Subsequently, blood derived monocytes migrate into the peritoneal space later on, between 24 hours and 5 days after challenge[186], where their role is to “clean up” the now apoptotic neutrophils, therefore helping to resolve inflammation and promote survival. We have previously reported that upon challenge with thioglycollate, there is an increased recruitment of neutrophils to the peritoneum of Fps-null mice [182], and have also shown that Fps-null mice display increased mortality upon an inflammatory challenge[67]. We therefore sought to investigate whether or not Fps-null mice properly resolved inflammation. To this end, peritoneal lavage cells from wild type and Fps-null mice were counted and analysed by flow cytometry, 5 days after i.p. challenge with thioglycollate. We observed that in the absence of Fps, there was a substantial decrease in the number of macrophages recruited to the peritoneal cavity (Figure 4-5). In fact, the number of cells recovered from the peritoneal cavities of Fps-null mice 5 days after thioglycollate challenge was actually less than the number recovered under resting conditions (compare numbers recovered in Figures 4-1 and 4-5). This suggested to us that Fps is necessary for the recruitment of monocytes to the peritoneal space following an inflammatory challenge. In the absence of Fps, the lack of recruitment of these “late” macrophages most likely hinders the resolution of inflammation. Indeed this seems to be the case, since 5 days after thioglycollate challenge, the percentage of neutrophils recovered from Fps-null mice was 2.5 times higher than the number recovered from wild type (3.3±0.3% vs. 8.1±0.2 %, P=0.002, wild type vs. fps⁻/⁻, Figure 4-5 inset).

It has been suggested that macrophage cell death upon pathogenic challenge may occur as a benefit to the host[195]. Therefore we propose that the observed disappearance of wild type macrophages subsequent to LPS challenge (Figure 4-1), may be due to increased cell death of
these cells (Figures 4-2 and 4-3), and that this ultimately promotes a proper resolution of inflammation (Figure 4-5); all of these processes appear to be defective in mice lacking Fps. Interestingly, a recent report by Moubarak et al. suggests that under certain conditions, cell death by necrosis can actually represent a form of programmed cell death [196], and not be a passive event as earlier thought. What is even more appealing about this idea is that they show that during this form of “programmed necrosis[196]” cells display phosphatidyl serine exposure and chromatin condensation, both of which we have used here to denote cells undergoing apoptosis (Figures 4-2, 4-3A). With this in mind, it is interesting to speculate that what we observed in Figures 4-2 and 4-3 is actually a form of TNF-α-induced programmed cell necrosis in macrophages, and not apoptosis, and that Fps is required for this process.

In conclusion, we show here that in the absence of Fps, there is an increased retention of macrophages in the peritoneal cavity subsequent to LPS challenge. We go on to show that this is likely due to a defect in TNF-α-induced death in macrophages lacking Fps. Furthermore, in the absence of Fps, we observed an increase in NF-κB signalling, and a decrease in PARP induction, both of which correlate with increased cell survival. Finally, we propose that this form of cell death, regulated by Fps, may be a programmed necrosis, which ultimately benefits the host by promoting the resolution of inflammation.
5.1 Summary of findings and significance of results

The \textit{fps} gene was originally identified as an oncogene from retro-virally induced chicken and avian tumours[1, 2]. Therefore, it is not surprising that much of the early work on \textit{fps}, and indeed much of the work done today, strives to elucidate what role this gene may have in the progression of malignant disease. Also, since early Fps expression was found to be highest in cells of the myeloid lineage, a large body of other work has focused on how \textit{fps} participates in the development and maturation of the hematopoietic system, giving particular attention to cells of the myeloid lineage. Due to the importance of myeloid (as well as other leukocytic, and endothelial) cells in innate immunity and inflammation, and the high expression of the Fps protein in these cells, it was not hard to imagine a role for Fps in these processes; however, it was not until a 2000 report by Hackenmiller \textit{et al.}[73], and a later more compelling 2002 report by Zirngibl \textit{et al.}[67], that \textit{fps} was conclusively shown to participate in innate immunity and inflammation. Each contained initial evidence that \textit{fps} might be important in the innate immune response, showing that their distinct Fps-knockout mouse models were more susceptible to \textit{B. burgdorferi}-induced footpad swelling, and death induced by intra-peritoneal LPS challenge, respectively. Nevertheless, both of these reports provided little evidence to suggest how the Fps protein was participating in inflammation. The only other clue as to what the role of Fps was in this process, was obtained by Senis \textit{et al.}[66], who showed that there was impaired LPS-induced signalling in macrophages obtained from mice harbouring a kinase-inactivating, knock-in mutation of \textit{fps}. To date, the Results chapters presented within this thesis are the only ones to focus solely on the role of \textit{fps} in inflammation and innate immunity. The work presented in this
thesis has expanded our knowledge of the role of the Fps protein tyrosine kinase in inflammation, by elucidating how it regulates this process at the physiological, cellular, and molecular levels.

5.1.1 Model of the role of Fps in inflammatory cytokine signalling and release.

One of the first occurrences in the response to injury, is the release of inflammatory cytokines by cells such as macrophages. These cytokines serve several functions, including the modulation of release of chemokines and cytokines, the up-regulation of adhesion molecules on leukocytes and epithelial cells, and the activation of cells of the innate and adaptive immune systems. Therefore, it was important to establish whether or not lack of a functional Fps protein could affect the release of, and signalling induced by, inflammatory cytokines.

In chapter two, we have shown that in the absence of Fps, there is an increased release of TNF-α into the serum of mice challenged with an intra-peritoneal LPS injection (Figure 2-1), and more specifically, that macrophages from these Fps-null mice showed increased LPS-induced secretion of TNF-α as well (Figure 2-2). This established that a major reason for the increased sensitivity of Fps-null mice to inflammatory challenge was due to increases in pro-inflammatory cytokine release from macrophages.

Release of cytokines from macrophages is directly linked to the increases in various signalling pathways that come about during the induction phase of inflammation. The major signalling pathway responsible for inducing the early release of TNF-α from macrophages is the NF-κB pathway. In chapter 2, we show that the role of Fps in macrophages is to down-regulate this pathway (Figure 2-3). Interestingly, TNF-α that has been released from macrophages, can act back on these cells in an autocrine manner. This autocrine action can further increase NF-κB signalling, leading to the release of additional inflammatory cytokines and chemokines from the macrophage. We therefore examined whether or not there may be differences in TNF-α-induced
signalling. Indeed, in chapter 4 we show that in addition to increased LPS-induced NF-κB activation, there is also increased TNF-α-induced NF-κB activation in macrophages isolated from Fps-null mice (Figure 4-5), although the latter is much more subtle. With this in mind, we propose a model in which Fps’ participation in LPS-induced signalling is on two levels; during both the initial LPS-induced TLR4 signalling, and subsequent autocrine TNF-α-induced TNFR1 signalling (Figure 5-1). Thus, the work mentioned above establishes Fps as an important modulator of the early, pro-inflammatory phase of inflammation, involving cytokine signalling and release.

5.1.2 Role of Fps in inflammatory cell recruitment and transmigration.

As mentioned above, the release of pro-inflammatory cytokines can stimulate the release of chemokines, and the up-regulation of cell-surface adhesion molecules. The ultimate goal of both of these processes is to allow for the early recruitment of inflammatory cells (mostly neutrophils) to a site of injury. These newly recruited neutrophilic cells can then combat infection at the source of injury; however, they can also inflict “collateral damage” to healthy tissues, due to the release of cytotoxic molecules such as reactive oxygen species. Therefore, an over abundance of neutrophils at a site of injury is a sign of excessive inflammation, which can lead to detrimental effects such as organ failure and death. With this in mind, in chapter 3 we examined the role of Fps in the in vivo recruitment of cells towards various inflammatory stimuli. In response to both LPS, and thioglycollate, there was an increase in the number of cells recruited in Fps-null mice (Figures 3-2B and 3-4A, respectively), and in the case of thioglycollate, we showed that these increases were due to a greater number of neutrophils being present (Figure 3-4B). This revealed a role for Fps in the recruitment of inflammatory cells during the early stages of inflammation, where it limits the response.
Figure 5-1: Model of Fps role in inflammatory signalling. Fps serves to control inflammatory signalling by restraining NF-kB activation after stimulation with LPS, or TNF-α. LPS signalling can lead to a feed forward autocrine loop involving TNF-α, and thus Fps can serve to regulate inflammatory signalling at two separate points in this signalling network pathway.
In contrast to the early stages of inflammation, during the later stages, monocytic cells are recruited to the site of injury, where their role is to phagocytose dead and dying neutrophilic and stromal cells, thereby promoting the resolution of inflammation. Again during this later stage, we observed differences in the recruitment of cells between Fps-null and wild type mice. In this instance, however, there was more cellular recruitment in wild type animals as compared to Fps-null, and these cells were predominantly macrophages (Figure 4-5). Therefore, our observations show that at times of both early and late cellular recruitment in response to inflammation, Fps-null mice display a phenotype that is in accordance with the promotion of inflammation. This suggests Fps can down-regulate inflammation by properly controlling cellular recruitment during inflammation, in addition to constraining the release of, and signalling downstream from, certain cytokines.

5.1.3 Possible role of Fps in non-innate immune cells in inflammation

Although this work focuses mainly on innate immune cell function in the context of inflammation, lymphocytes are also involved in sepsis. During sepsis, there is extensive lymphocyte apoptosis [197], and this probably contributes to the immunosuppression seen in many septic patients [100]. Furthermore, T cells can sequester cytokines in the resolution phase of inflammation[198]. Therefore, it would be interesting to investigate a possible role for Fps in inflammation with respect to lymphocytic immune cells, since loss of Fps leads to slight perturbations in the levels of B cell progenitors[67], albeit independent of its kinase function [66].

Fps is also expressed in platelets. Platelets are important in coagulation, and indeed platelets from Fps and Fer deficient mice displayed disregulated platelet aggregation and disaggregation, respectively [35]. Furthermore, whole blood from Fps-null mice displayed a reduced thrombin clot time, as well as increased Fibrin content (Parsons, Sangrar and Greer, unpublished observations). With respect to inflammation, this is important since sepsis very
frequently leads to the occurrence of disseminated intravascular coagulation (DIC) [199]. DIC can be defined as the activation of intravascular coagulation or the appearance of microthrombi, and this syndrome most likely contributes to the multiple organ failure associated with sepsis, due to inadequate tissue perfusion [100, 199, 200]. Interestingly, it has been shown that in the \( fps^{MF} \) mouse model, a DIC-like syndrome occurs naturally [201].

5.1.4 Effects of Fps on apoptosis in macrophages, and subsequent secretion of cytokines.

A vital part of the resolution phase of inflammation, is the ability of inflammatory cells to undergo apoptosis, thereby limiting the tissue damage caused by these cells. A great deal of the work done to date has focused on the role of neutrophil apoptosis in the resolution of inflammation[118, 121, 122]. Here, we show that in the absence of Fps, macrophages are more resistant to LPS-induced inflammation, while mice lacking Fps are more susceptible to it. While studies seem to show that an increased rate of macrophage apoptosis relative to control is detrimental to the host in the setting of inflammation, this does not necessarily mean that a decrease relative to control, as observed in chapter 4, would be beneficial. In fact, one study suggests that inhibition of apoptosis with the pan-caspase inhibitor Z-VAD-FMK (zVAD), correlates with an increase in the LPS-induced release of the pro-inflammatory high mobility group box protein 1 (HMGB1)[202]. However, HMGB1 is released relatively late in the context of an LPS challenge, relative to other pro-inflammatory cytokines such as TNF-\( \alpha \) (about 20 hours after stimulation versus 1-2 hours after stimulation, respectively). To our knowledge, little work has been done on the role of apoptosis in modulating the early response of macrophages to stimulation with pro-inflammatory molecules such as LPS, and their subsequent ability to release cytokines. Our results show that there is a correlation between a reduction in apoptosis in peritoneal macrophages, and an increase in their ability to secrete cytokines (compare data from Figures 2-2, and 4-2). We postulate that this correlation could exist for two reasons. First,
decreased apoptosis in Fps-null cells as compared to wild type, would allow for an increased length of time over which they could secrete cytokines, thereby allowing for an increase in the amount of secretion. Secondly, this correlation could be a bi-product of these two events being stimulated by some of the same signalling pathways. For example, activation of NF-κB will lead to increases in the transcription (and presumably the secretion) of cytokines such as TNF-α[138], but has also been shown to stimulate cellular survival[188]. With this in mind, it would be interesting to investigate the secretion of early-released cytokines such as TNF-α or IL-1β, from macrophages cultured with and without apoptosis inhibitors such as zVAD.

5.1.5 A proposed mechanism by which Fps can function in the cell.

This work has demonstrated three main functions for the Fps protein. In the second and fourth chapters, we show a function for Fps in signalling downstream of both LPS and TNF-α. In chapter 2, we provide evidence of a role for Fps in endocytosis, and possibly phagocytosis as well. Finally, in chapters 3 and 4, we show a role for Fps in regulating cellular recruitment in response to inflammatory stimuli. Although these processes seem to be very different in nature, they share a common thread through which Fps might act upon them all.

Fps is a member of the *Pombe* Cdc15 homology (PCH) family of proteins, whose characteristic feature is an evolutionarily conserved extended FCH (EFC) or FCH-BAR (F-BAR) domain, located on the N-terminus of these proteins[21, 22]. The structure of the EFC domain allows it to bind to curved phospholipid membranes, where it can participate in the regulation of clathrin-mediated endocytosis (CME)[23]. Presumably, most of this family of proteins participate in CME, through the recruitment of dynamin to their Src homology 3 (SH3) domains[13, 203], thereby allowing dynamin to carry out its vesicle scission function[204-206]. As well, at least one of these PCH proteins also recruits neural Wiskott-Aldrich syndrome protein (N-
WASP) [207], which is also necessary for CME, and participates in actin cytoskeletal dynamics in general. Therefore, it is tempting to suggest that the Fps protein might play a similar role in regulating CME, and actin cytoskeleton dynamics in general. As discussed in chapter 2, there is preliminary evidence for such a role. Briefly, the Fps-related Fer kinase might participate in actin cytoskeleton function through regulation of cortactin phosphorylation [86, 88-90]. Fps and Fer are highly homologous [85], thus these kinases may have similar or redundant roles within the cell, implicating Fps in the regulation of the actin cytoskeleton. Our preliminary results suggest a role for Fps in regulating phosphorylation of HS-1, a cortactin homolog. Interestingly, HS-1 has been shown to participate in the regulation of actin cytoskeletal dynamics, by binding to both F-actin and the Arp2/3 complex, which is mediated by its coiled-coil and N-terminal acidic domains, respectively [208, 209]. The binding of HS-1 to the Arp2/3 complex, increases the rate of actin assembly by the later, as well as its ability to promote actin branching [209]. More recent work by Udell and Craig suggest that tyrosine phosphorylation of HS-1 is reduced in Fps and Fer deficient mast cells, which have been activated via FcεR1 (Udell and Craig, unpublished observations). Therefore, it is interesting to speculate that Fps might participate in actin re-organization through HS-1 phosphorylation, much in the same was as Fer does with cortactin.

Secondly, recent work by Laurent et al. showed a role for human Fps in the regulation of the tubulin cytoskeleton [15], which is also involved in endocytosis (reviewed in [160]). Also, Fps partially co-localized with Rab5B and Rab7, suggesting a role in vesicular trafficking [20], which is another event involving actin re-organization [210].

In this thesis, we show that Fps is involved in regulating at least three separate events in inflammation: cytokine release, cellular recruitment, and apoptosis. Actin re-organization is important for all three of the processes. Cytokine release is affected by the regulation of receptor endocytosis [139], which requires actin re-organization [140]. In turn, receptor endocytosis is important for TNF-α-induced apoptosis [189]. Finally, recruitment of cells to sites of inflammation, involve various events, many of which rely upon the dynamic nature of the
cytoskeleton in order to function properly[173, 179]. Therefore, we propose that it is through its proposed ability to regulate the cytoskeleton, that the Fps protein can regulate all three of the events described above.

5.2 Effect of inflammation on tumour biology, and possible roles for the Fps protein.

The idea of inflammation being associated with cancer is not new, and scientists have been studying the question for over a century and a half, and the relationship between the two is the subject of many review articles[78, 79, 211, 212]. Different types of inflammation can affect cancer development in different ways; simply put, acute inflammation tends to be anti-oncogenic, while chronic inflammation tends to be oncogenic[213]. In fact, many individual cancers have been associated with various specific types of chronic inflammation. For example, *H. pylori* infection is strongly correlated with the appearance of gastric cancer[214], Hepatitis C infection has been correlated with hepatocellular carcinoma, and Crohn’s disease and ulcerative colitis seem to promote the induction of colon carcinoma. In fact, it has been reported that there is a reduction in colon cancers among long term users of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, by up to 40%[215, 216].

One of the ways in which chronic inflammation can promote cancer progression, is through the neo-vascularization of tumours. Macrophages that are associated with tumours, or TAMs, can produce large amounts of TNF-α in the local tumour environment. In some instances, this can then stimulate the release of VEGF-A from melanocytes, thereby promoting the vascularization of tumours[216, 217]. It is interesting to speculate that there might be a role for Fps in this process, since Fps-null macrophages release increased amounts of TNF-α[170], and fps<sup>mf</sup> endothelial cells show an enhanced sensitivity to VEGF stimulation[62]. Therefore, future studies could examine the extent to which xenografted tumours can induce neovascularization, in the context of nude mice harbouring various genetic mutations of the fps gene.
Inflammation can also exert an effect on tumour infiltration and metastasis. It is well established that in an inflammatory setting, the release of specific chemokines, such as GROα, GROβ, and IL-8 can lead to the recruitment of leukocytes to the site of injury; however, these same molecules can also exert direct stimulatory effects on tumours[218]. P-selectin, another important molecule in leukocyte recruitment, which is up-regulated during inflammation, can participate in the metastasis of melanoma cells to the lung[219]. Furthermore, recent work by Wyckoff et al. describes a role for perivascular macrophages in assisting the intravasation of tumour cells, a process that is necessary for hematogenous metastasis[220]. With this in mind, it would be interesting to investigate the possibility of a role for Fps in regulating tumour metastasis, especially when one considers the role shown here for this protein in leukocyte recruitment.

Overcoming natural apoptotic regulation is one of the six hurdles that a nascent tumour must leap in order to become fully carcinogenic[221]. In the context of inflammation, the release of TNF-α can have a dramatic effect on this process. In locally administered high doses, TNF-α can stimulate the regression of tumours[222]; however, in other cases this molecule seems to have the opposite effect [223]. It has been suggested that the tumour promotion effect of TNF-α, most likely arises from its ability to stimulate the pro-survival NF-κB pathway. Indeed, the constitutive activation of this pathway has been linked to the promotion of cancers, especially those of epithelial origin[212, 224]. Sangrar et al. have shown that in a mouse model of breast epithelial cancer, tumour onset time is reduced in both Fps-null, and $fps^{K>R/K>R}$ mice. Considered alongside the evidence presented in this thesis which suggests that an intact Fps protein can restrain inflammation-induced NF-κB activity (Figures 2-3 and 4-4), one might theorize that the role of Fps in restraining tumour onset, is at least partially due to its ability to regulate NF-κB activity in the context of inflammation.
During inflammation, the production and release of reactive oxygen species (ROS) and reactive nitrogen species (RNS), is one of the ways in which leukocytes attempt to rid the body of foreign pathogens. The release of ROS and RNS can induce mutations in DNA, since these two groups of molecules can react to produce peroxynitrite, a known mutagen[225]. Interestingly, Fps and Fer can be inducibly phosphorylated by ROS[226] (Parsons and Greer, unpublished observations), and at least with respect to Fer, some of its functions seem to be dependent on the generation of these molecules[227]. Therefore, future investigation could explore the possibility that ROS-induced phosphorylation of Fps may in some way affect tumour generation and progression.
References


