INVESTIGATION OF NOVEL SIGNALS INDUCED BY LIPOPOLYSACCHARIDE IN INNATE IMMUNE CELLS

by

SHERIE KRISTINA DUNCAN

B.Sc., Simon Fraser University, 2004

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(Experimental Medicine)

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Abstract

Macrophages play key roles in innate immune responses. They phagocytose and destroy bacteria, and are activated by various microbial products, recognized through a variety of pattern recognition receptors, including toll-like receptors (TLRs). Engagement of TLRs triggers various intracellular signals, leading to the release of mediators that help orchestrate both the innate and adaptive immune responses. Deficiency in the ability of the host to detect and respond to bacterial products leads to repeated or chronic infections, whereas excessive responses to bacterial products can lead to septic shock and death.

Lipopolysaccharide (LPS) is a major constituent of the cell wall of gram-negative bacteria and a chief contributor to the development of septic shock in infections with gram-negative bacteria. The current paradigm is that all biological responses to LPS from *Escherichia coli* depend on TLR4. Here, we refute this paradigm and present evidence demonstrating that alternate receptors, in addition to TLR4, transduce signals from LPS in macrophages. Using ultra-pure preparations of LPS, we found that activation of p21Ras and phosphorylation of Akt and Src family kinase targets such as Cbl, Pyk2, Vav and Syk occurred even in absence of TLR4, whereas MAPK activation was completely dependent on TLR4. CD14 and class A (SR-AI/II) and class B (CD36) scavenger receptor members were implicated in TLR4-independent signalling, indicating that these LPS-binding proteins play a greater role in pathogen recognition than previously appreciated. We identified downstream pathways
mediated by TLR4-independent signalling that include promotion of cellular viability, changes in cell morphology and induction of gene expression, most notably Il1a and Il1b expression. We also observed changes in leukocyte distribution in TLR4-deficient animals following LPS treatment, confirming that TLR4-independent pathways also function *in vivo*. We observed that TLR4 signalling negatively regulated the activation of p21Ras and Cbl, indicating that the TLR4-independent and TLR4-dependent pathways influence one another. Furthermore, we found that the negative effect of TLR signalling on p21Ras activation required MAPK activity and also affected cytokine signalling. We propose that the TLR4-independent pathway provides signals that are critical for the development of a global response to infection and may represent a general mechanism for sensing pathogens.
Preface

Authorship Statement

Chapter 3

All experiments were designed, performed and analyzed by the author with the exception of Figure 3.1B, which was designed, performed and analyzed by Dr. Christopher Cochrane. Dr. Muriel David provided assistance in the design and analysis of experiments included in Figures 3.1A, 3.1B, 3.2 – 3.11. Dr. Kyla Omilusik provided assistance with flow cytometry in Figure 3.1C.

Figures 3.1A, 3.1B and 3.2 are included in the publication David, M.D., C.L. Cochrane, S.K. Duncan and J.W. Schrader. *Pure Lipopolysaccharide or Synthetic Lipid A Induces Activation of p21Ras in Primary Macrophages through a Pathway Dependent on Src Family Kinases and PI3K*. Journal of Immunology, 2005(175): 8236-8241.

Chapter 4

All experiments were designed, performed and analyzed by the author with the exception of Figure 4.1A, which was designed, performed and analyzed by Dr. Muriel David. Dr. David also provided assistance in the design and analysis of experiments included in Figures 4.1, 4.3A, 4.3B, 4.3D and 4.5A. Dr. D. James Haddon provided assistance in the experimental design in Figure 4.7. Dr. Welso
Wang (intraperitoneal injections) and Mr. Matthew Gold (flow cytometry) provided technical assistance with the experiments in Figure 4.7.

Figures 3.4 – 3.13, 4.1 – 4.5 and 4.7 are prepared in a manuscript for submission [Sherie K. Duncan, Muriel D. David and John W. Schrader (2014) Lipopolysaccharide from Escherichia coli induces Toll-like receptor 4-independent responses that are mediated by CD14 and scavenger receptors].

Chapter 5
All experiments were designed, performed and analyzed by the author. Dr. Muriel David provided assistance with the design and analysis of experiments included in Figures 5.1 – 5.3 and 5.6A.

Chapter 6
All experiments were designed, performed and analyzed by the author. Dr. T. Michael Underhill provided assistance with the analysis of the microarray data included in Figure 6.1 and Table 6.1. Dr. Yanni Wang provided technical assistance with qRT-PCR for experiments included in Figures 6.2 – 6.4.

Animal Care Protocol
All studies were performed following the guidelines set by both the University of British Columbia’s Animal Care Committee and the Canadian Council on Animal Care. The animal care protocol for these experiments was A13-0039.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAM</td>
<td>alternatively activated macrophages</td>
</tr>
<tr>
<td>ACK</td>
<td>ammonium-chloride-potassium</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<td>AP-1</td>
<td>activation protein 1</td>
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<td>activating transcription factor 2</td>
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<td>B cell lymphoma 2</td>
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<td>BM</td>
<td>bone marrow</td>
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<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>B4</td>
<td>0111:B4</td>
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<td>CAM</td>
<td>classically activated macrophages</td>
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<td>caspase</td>
<td>cysteine-dependent aspartate specific protease</td>
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<td>Cbl</td>
<td>Casitas B-lineage lymphoma</td>
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<td>Ccl</td>
<td>chemokine (cysteine-cysteine motif) ligand</td>
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<td>Ccr1</td>
<td>chemokine (cysteine-cysteine motif) receptor-like</td>
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<td>CD</td>
<td>cluster of differentiation</td>
</tr>
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<td>cDNA</td>
<td>complimentary DNA</td>
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<td>cIAP2</td>
<td>cellular inhibitor of apoptosis 2</td>
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<td>COX-2</td>
<td>cyclooxygenase 2</td>
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<td>CpG</td>
<td>cytosine-phosphodiester bond-guanine</td>
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<td>CrkL</td>
<td>chicken tumor virus number 10 regulator of kinase-like protein</td>
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<td>CREB</td>
<td>cyclic adenosine monophosphate response element-binding protein</td>
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<td>CSF</td>
<td>colony-stimulating factor</td>
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<td>CSF-1R</td>
<td>CSF-1 receptor</td>
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<td>CT</td>
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<td>DAMP</td>
<td>damage-associated molecular patterns</td>
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<td>Dulbecco’s modified Eagle media</td>
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<td>double-stranded RNA</td>
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<td>E. coli</td>
<td>Escherichia coli</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>ELISA</td>
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<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>EtN</td>
<td>ethanolamine</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>Fc</td>
<td>fragment, crytallizable</td>
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<tr>
<td>FcγR</td>
<td>Fc-gamma receptor</td>
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<tr>
<td>Fpr1</td>
<td>N-formyl peptide receptor</td>
</tr>
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<td>FSC</td>
<td>forward scatter</td>
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<td>GAP</td>
<td>GTPase-activating protein</td>
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<tr>
<td>gbw</td>
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<td>GEF</td>
<td>guanine-nucleotide exchange factor</td>
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<td>GM-CSF</td>
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<td>glutathione S-transferase</td>
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<td>GTPase</td>
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<td>HBSS</td>
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<td>heat shock protein</td>
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<td>interferon-induced protein with tetratricopeptide repeats</td>
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<td>LTA</td>
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MAL  MyD88-adaptor-like
MAPK  mitogen-activated protein kinase
MD2  myeloid differentiation protein 2
MEK  mitogen-activated protein kinase kinase
MFI  mean fluorescent intensity
MyD88  myeloid differentiation primary-response protein 88

NEAA  nonessential amino acids
NEMO  NFκB essential modulator
NFAT  nuclear factor of activated T cells
NFκB  nuclear factor of kappa polypeptide enhancer in B cells
Nfkbiz  NFκB inhibitor, zeta
NGa  N-acetyl-galactosamine
NGc  N-acetyl-glucosamine
NOD  nucleotide-binding oligomerization domain

ODN  oligodeoxynucleotides

PAGE  polyacrylamide gel electrophoresis
PAMP  pathogen-associated molecular pattern
PARP  poly ADP-ribose polymerase
PBS  phosphate-buffered saline
PI3K  phosphatidylinositol 3-kinase
PKB  protein kinase B
PKC  protein kinase C
PLC  phospholipase C
PMA  phorbol 12-myristate 13-acetate
PMB  polymyxin B
poly(I:C)  polyniosinic:polycytidylic acid
PRR  pattern recognition receptor
Ptgs2  Prostaglandin-endoperoxide synthase 2
PYK2  proline-rich tyrosine kinase 2

qRT-PCR  quantitative reverse-transcription polymerase chain reaction

RAGE  receptor for advanced glycation end products
RANTES  regulated on activation, normal T cell expressed and secreted
Ras  rat sarcoma
Rassf4  Ras association (RalGDS/AF-6) domain family member 4
RBC  red blood cells
RBD  ras-binding domain
RIG-I  retinoic acid-inducible gene 1
RIP1  receptor-interacting protein 1
RPII  RNA polymerase II
RPMI  Roswell Park Memorial Institute
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<tr>
<td>Rsad2</td>
<td>Radical S-adenosyl methionine domain-containing 2</td>
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<td>SR-AI/II</td>
<td>scavenger receptor class A type I/II</td>
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<td>SD</td>
<td>standard deviation</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>src family kinase</td>
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<td>TAK1</td>
<td>TGF-β activated kinase 1</td>
</tr>
<tr>
<td>TARM1</td>
<td>T cell-interacting, activating receptor on myeloid cells 1</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK-binding kinase 1</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>Tet2</td>
<td>Tet methylcytosine dioxygenase 2</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Tgtp1</td>
<td>T cell specific GTPase 1</td>
</tr>
<tr>
<td>TICAM-1</td>
<td>TIR-containing adaptor molecule-1</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-interleukin 1 receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR domain-containing adaptor protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF receptor type 1-associated death domain protein</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associate factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain-containing adaptor protein inducing interferon beta</td>
</tr>
<tr>
<td>Upp1</td>
<td>uridine phosphorylase 1</td>
</tr>
<tr>
<td>Viperin</td>
<td>virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible</td>
</tr>
<tr>
<td>WST-1</td>
<td>water soluble tetrazolium salt 1</td>
</tr>
<tr>
<td>Zbp1</td>
<td>Z-DNA binding protein 1</td>
</tr>
<tr>
<td>βc</td>
<td>common β chain of GM-CSF, IL-3 and IL-5</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin D</td>
</tr>
</tbody>
</table>
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Dedication

To my family

Tracey Deschênes – my sister, my best friend, the person I can always, always rely upon.

Bruce Duncan – my father, who never gives himself enough credit for taking on such an incredible task.

Whenever I succeed, it’s because I know you are both there, cheering me on. Your love and encouragement means everything to me.

And to Judy Duncan – my mother, gone much too soon, but I like to think you would be proud.
Chapter 1. General introduction

Vertebrates have evolved a complex, multi-layered defence system to protect against a wide range of infectious agents [1]. This defence system is comprised of two major components: the innate and adaptive immune systems [2]. Innate immune responses are characterized by rapid recognition and mobilization against invading pathogens [1]. Cells of the innate immune system are activated through a series of germ-line encoded receptors termed pattern recognition receptors (PRRs), which recognize molecular structures that are evolutionarily conserved across many species of pathogens. These conserved structures are known as pathogen-associated molecular patterns (PAMPs) [3, 4]. However, the innate immune system is constrained by its reliance on a limited and invariant repertoire of PRRs [1, 4].

Pathogens that bypass, overwhelm or escape innate immune effectors encounter the adaptive immune system. Although adaptive immunity takes days to develop, cells of the adaptive immune system, such as B cells and T cells, generate a large repertoire of pathogen-specific receptors by rearranging gene segments. In this manner, adaptive immune cells may recognize an almost infinite variety of antigens and consequently tailor the immune response specifically to the invading pathogen [1, 5]. In addition, after elimination of the pathogen, the adaptive immune system provides long-term protection from subsequent re-infection of the same pathogen in the form of immunological memory [1].
The innate and adaptive immune systems complement one another both functionally and temporally. Cells of the innate system act as sentinels, identifying foreign agents and mounting an immediate first-line response [1]. This allows time for adaptive immune response to develop, poised and ready to respond if the defences of the innate immune system are breached.

Effector cells of the innate immune system such as macrophages, monocytes and granulocytes contribute to both innate and adaptive responses [4]. As part of the innate response, these cells are among the first to identify and respond to pathogens. They use phagocytosis as a key effector mechanism to uptake and destroy pathogens [6]. They also secrete chemical mediators that recruit additional effector cells and direct the development of an appropriate adaptive immune response [5]. In addition, some innate immune cells, such as macrophages, are used as effector cells during adaptive responses [4]. Thus, the co-operation of the innate and adaptive immune systems ensures protection against a large variety of infectious agents.

1.1 Macrophages

Macrophages are large, mononuclear phagocytic cells that reside in the tissues. Some classification systems also consider monocytes to be a type of mobile macrophage [7]. The ability to phagocytose particles is a defining characteristic of all macrophages and monocytes [7]. Macrophages were first described in the late 19th century when their recruitment to sites of infection and their ability to engulf microbes was noted (reviewed in [8]). During these early studies, macrophages were observed in many tissues of the body,
including lymph nodes, spleen, liver, lung and intestine. Since then, macrophages have been identified in nearly all tissues of the body and have been shown to have a variety of functions in addition to their role in immune responses. Macrophages are critical participants during development, in tissue homeostasis and wound healing, among others (reviewed in [9-11]). Figure 1.1 summarizes some of the major tissues in which macrophages can be found, and some of their identified roles in those tissues.

Figure 1.1. Tissue resident macrophages: location and function. Macrophages can be found in most tissues and have specialized functions specific to their tissue of residence. Figure from [12] and reprinted with permission from www.macrophages.com, The Roslin Institute, The University of Edinburgh, Copyright 2012.
1.1.1 Macrophages in immune responses

Macrophages are an exceedingly heterogeneous cell population, a consequence of their specific microenvironment and the specialized functions they have in different tissues [10, 13]. However, a common function among many tissue-resident macrophage populations is that of a first line of defence against invading microorganisms. Following recognition of the pathogen through PRRs, tissue-resident macrophages will engulf and attempt to kill invading microorganisms [6]. Macrophages activated by PAMPs secrete pro-inflammatory cytokines, chemokines and other biologically active molecules, inducing inflammation. This results in the recruitment of additional inflammatory cells, both innate and adaptive, to the infected tissues [14, 15].

Macrophages also play a key role in adaptive immune responses [13]. As professional antigen presenting cells, macrophages display degraded pathogenic products at the cell surface for activation of antigen-specific T cells [6]. As well, the cytokines released by activated macrophages help direct the development of the appropriate adaptive immune response [5]. Finally, the phagocytic capacity of macrophages is exploited as an effector mechanism of the adaptive immune response. Antibodies released by plasma cells opsonize pathogens, thereby increasing the efficiency of their phagocytic uptake by macrophages [6].

1.1.2 Macrophage subpopulations

Macrophages are generally divided into two distinct classes: tissue-resident macrophages and infiltrating macrophages. As the name suggests, tissue-resident macrophages are
found in the tissues (Figure 1.1) and play critical roles in local tissue homeostasis during steady state conditions [16]. For many years, monocytes were thought to contribute to all macrophage populations [7]. However, many tissue-resident macrophage populations are established before embryonic appearance of monocytes, including macrophages in the brain, skin, liver, lung, spleen and peritoneal cavity [17-19]. Recent studies indicate that these populations locally self-maintain with minimal contribution from circulating monocytes [17, 19, 20]. In contrast, monocytes do contribute to some populations of tissue-resident macrophages in the gastrointestinal tract [21] (Figure 1.2A).

Infiltrating, or inflammatory, macrophages are recruited to the tissues following inflammation elicited by infection, damage or disease [16]. These macrophages support the local tissue resident macrophage population during inflammatory conditions and are derived from circulating monocytes that are recruited to sites of inflammation where they then differentiate into macrophages [22]. Infiltrating macrophages are ultimately derived from hematopoietic stem cells in the bone marrow [22] (Figure 1.2B).
Figure 1.2. Origins of tissue-resident and infiltrating macrophages.

(A) Many tissue-resident macrophage populations are established prior to birth. Some populations are seeded by phagocytes derived from the yolk sac, while others are seeded after the establishment of hematopoiesis in fetal liver or bone marrow. Recent evidence suggests that these populations self-maintain under steady-state conditions and undergo local proliferation to restore homeostatic tissue-resident macrophage populations following pathology. (B) Under inflammatory conditions, circulating monocytes are recruited to the tissues where they are induced to differentiate into macrophages known as infiltrating macrophages. These cells are ultimately derived from hematopoietic stem cells. Figure 1.2A modified from [23] and used with permission from Nature Publishing Group, Copyright 2013.

Further subdivision of macrophage populations can be made following phenotypic polarization by the local microenvironment. Polarized macrophages are distinguished by cell-surface markers and gene expression profiles and are broadly separated into two categories: (1) classically activated macrophages (CAM) or M1 macrophages; and (2)
alternatively activated macrophages (AAM) or M2 macrophages (reviewed in [24] and [16]) (Figure 1.3).

![Diagram of polarized macrophages]

**Figure 1.3. Characteristics of polarized macrophages.**
Classically activated macrophages (M1) are activated by microbial products and produce inflammatory cytokines and reactive oxygen and nitrogen intermediates. Alternatively activated macrophages (M2) are activated by IL-4/IL-13 (M2a), immune complexes and microbial products (M2b) or IL-10 and glucocorticoids (M2c). Arginase production is characteristic of M2 macrophages. *Figure modeled on figure from [25] and used with permission from The American Association of Immunologists, Inc., Copyright 2008.*

CAM/M1 macrophages arise in microenvironments rich in microbial products and interferons, and they are microbicidal and inflammatory [25]. Tissue destruction is a hallmark of CAM/M1 macrophages as a consequence of the pro-inflammatory cytokines, chemokines and proteolytic enzymes released by this type of macrophage [26].
AAM/M2 macrophages, on the other hand, are immunomodulators. They are poorly microbicidal and play a role in the resolution of inflammation [25]. AAM/M2 macrophages are further subdivided into three additional subsets: M2a (induced by interleukin 4 (IL-4) or IL-13), M2b (induced by immune complexes in combination with microbial products or IL-1 stimulation) and M2c (induced by IL-10, transforming growth factor beta (TGF-β) or glucocorticoids) [24, 25]. Although these categories of macrophage polarization are generally thought of as being distinct states, there is abundant evidence that these phenotypes are plastic and can change depending on the type, concentration and duration of exposure of stimulating agents in the microenvironment [27-33]. Thus, the same macrophage population may take part in both inflammation and its resolution [34].

1.2 Toll-like receptors

There are many different classes of PRRs that recognize a wide diversity of PAMPs from microorganisms such as bacteria, viruses, parasites and fungi. Mammalian PRRs are generally divided into two classes: membrane-bound receptors, such as the families of Toll-like receptors (TLRs) and C-type lectin receptors, and cytoplasmic receptors, which include the nucleotide-binding oligomerization domain (NOD)-like receptors, retinoic acid-inducible gene 1 (RIG-I)-like receptors and other cytoplasmic nucleic acid sensors [35]. TLRs were the first PRRs to be identified and are among the best characterized [36].

TLRs are homologs of the receptor Toll in invertebrates. Toll was first identified as a gene important for specifying correct dorso-ventral patterning in Drosophila melanogaster [37]. Later, Toll was also found to play a role in immune responses to fungal [38] and bacterial
pathogens through recognition of fragments of the *Drosophila* protein, Spätzle, which is cleaved following infection. Initiation of Toll signalling leads to activation of the Rel family of transcription factors and induction of gene expression [40]. Similarly, ligand binding to TLRs triggers signalling pathways that result in activation of the nuclear factor of kappa polypeptide enhancer in B cells (NFκB)/Rel family of transcription factors in mammals [36]. Thus, TLRs represent an ancient and highly conserved pathogen recognition pathway.

TLRs are present in great diversity in vertebrates and invertebrates. Thirteen TLR family members have been identified that are expressed in humans and/or mice that recognize lipid, lipoprotein, protein and nucleic acid PAMPs in the extracellular compartment or intracellular vesicles [36, 41]. In addition, endogenous ligands have been identified for some TLRs. These endogenous ligands, termed damage-associated molecular patterns (DAMPs), are mediators that are released under situations of cellular stress or damage. These include degradation products of the extracellular matrix, heat-shock proteins, chromatin-DNA and ribonucleoprotein complexes, among others [36].

TLRs can be grouped based on their cellular localization and the PAMPs they recognize. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on cell surfaces and recognize microbial membrane components, such as lipids, lipoproteins and proteins. TLR3, TLR7, TLR8, TLR9 and TLR13, on the other hand, are expressed exclusively on intracellular vesicles, such as endoplasmic reticulum, endosomes, lysosomes and endolysosomes, and recognize microbial nucleic acids [36]. TLR11 and TLR12 are also expressed on intracellular vesicles, but recognize microbial proteins [41]. The cellular localization and
ligands for TLR10 are currently not known [41, 42]. Correct cellular localization of TLRs is particularly important to ensure accessibility to their ligands and also to maintain tolerance to self-molecules that otherwise might bind and activate TLR signalling pathways [36].

Table 1.1 lists the 13 TLRs that have been identified in human or mouse, their ligands (both PAMPs and DAMPs), adaptors and cellular localization.
Table 1.1. TLRs: ligands, localization, adaptors and species.

<table>
<thead>
<tr>
<th>Name</th>
<th>PAMPs</th>
<th>DAMPs</th>
<th>Cellular localization</th>
<th>Adapter(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Triacyl lipoproteins (with TLR2)</td>
<td>N.D.</td>
<td>Plasma membrane</td>
<td>MyD88/MAL</td>
</tr>
<tr>
<td>TLR2</td>
<td>Lipoproteins: triacyl lipoproteins (with TLR1); diacyl lipoproteins, LTA &amp; zymosan (with TLR6)</td>
<td>HMGB1, HSPs, ECM (with TLR6)</td>
<td>Plasma membrane</td>
<td>MyD88/MAL</td>
</tr>
<tr>
<td>TLR3</td>
<td>dsRNA</td>
<td>mRNA</td>
<td>Endosomal/lysosomal membrane</td>
<td>TRIF</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS, viral envelope proteins</td>
<td>HMGB1, HSPs, ECM, oxidized phospholipids, β-defensin 2; amyloid-β &amp; oxidized LDL (with TLR6)</td>
<td>Plasma membrane &amp; endosomal/lysosomal membrane</td>
<td>MyD88/MAL TRIF/TRAM</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td>N.D.</td>
<td>Plasma membrane</td>
<td>MyD88/MAL</td>
</tr>
<tr>
<td>TLR6</td>
<td>Diacyl lipoproteins, LTA &amp; zymosan (with TLR2)</td>
<td>HMMGB1, HSPs, ECM (with TLR2)</td>
<td>Plasma membrane</td>
<td>MyD88/MAL</td>
</tr>
<tr>
<td>TLR7</td>
<td>ssRNA</td>
<td>ssRNA immune complexes</td>
<td>Endosomal/lysosomal membrane</td>
<td>MyD88</td>
</tr>
<tr>
<td>TLR8</td>
<td>ssRNA</td>
<td>ssRNA immune complexes</td>
<td>Endosomal/lysosomal membrane</td>
<td>MyD88</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG DNA, hemozoin</td>
<td>DNA immune complexes</td>
<td>Endosomal/lysosomal membrane</td>
<td>MyD88</td>
</tr>
<tr>
<td>TLR10</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>TLR11</td>
<td>Profilin-like protein, Uropathogenic bacteria</td>
<td>N.D.</td>
<td>Endosomal/lysosomal membrane</td>
<td>MyD88</td>
</tr>
<tr>
<td>TLR12</td>
<td>Profilin</td>
<td>N.D.</td>
<td>Endosomal/lysosomal membrane</td>
<td>MyD88</td>
</tr>
<tr>
<td>TLR13</td>
<td>Bacterial RNA (23S rRNA)</td>
<td>N.D.</td>
<td>Endosomal/lysosomal membrane</td>
<td>MyD88</td>
</tr>
</tbody>
</table>

TLRs 1-9 are expressed in both human and mice. TLR10 is expressed only in humans. TLRs 11-13 are expressed only in mice. Abbreviations: dsRNA, double-stranded RNA; ECM, extracellular matrix; HMGB1, high mobility group box 1; HSPs, heat shock proteins; LPS, lipopolysaccharide; LTA, lipoteichoic acid, N.D., not determined; ssRNA, single-stranded RNA. Data compiled from [36, 41, 42].

1.2.1 TLR signalling

TLRs are Type I transmembrane proteins composed of three major regions: (1) an ectodomain containing leucine-rich repeats that mediate recognition of ligands; (2) a
transmembrane domain; and (3) an intracellular Toll-interleukin 1 receptor (TIR) domain that is required for downstream signal transduction [36, 43].

TLRs are thought to signal as dimers [43]. Many TLRs function primarily as homodimers, although some TLRs, particularly TLR1, TLR2 and TLR6, function as heterodimers (TLR1/2 or TLR2/6) with ligand specificity differing for each dimer [44, 45]. In addition, some TLRs require the participation of other accessory proteins for optimal ligand sensitivity [46]. For example, MD-2, an unanchored protein that associates with TLR4 on the cell surface, is an essential component of the TLR4 signalling complex [47, 48].

Ligand binding to the TLR signalling complex triggers the selective recruitment of distinct cytoplasmic adaptors through TIR-TIR domain interaction between the receptor and TIR domain-containing adaptor proteins (Figure 1.4). The adaptors that are recruited to the TIR domain determine the specificity of the signalling pathway [49]. The adaptor protein myeloid differentiation primary-response protein 88 (MyD88) is used by all TLRs except TLR3. Initiation of MyD88-dependent signalling generally leads to activation of mitogen-activated protein kinases (MAPKs) and the transcription factor NFκB, promoting the production of inflammatory cytokines [50, 51]. Although other TLRs are thought to recruit the TIR domain of MyD88 directly to their TIR domains, TLR2 and TLR4 instead use the TIR domain-containing adaptor protein (TIRAP), also known as MyD88-adaptor-like (MAL), to recruit MyD88 to the TLR signalling complex [52-54].
TLR3, on the other hand, uses the adaptor TIR domain-containing adaptor protein inducing interferon beta (TRIF), which is also known as TIR-containing adaptor molecule 1 (TICAM1). The TRIF-dependent pathway activates the interferon regulatory factor (IRF) transcription factors, promoting the production of type I interferon, while also activating MAPKs and NFκB to promote the production of inflammatory cytokines [55, 56]. TLR4 can also activate the TRIF-dependent pathway, recruiting TRIF through the adaptor TRIF-related adaptor molecule (TRAM) after trafficking to the endosome [57, 58].
Figure 1.4. PAMP recognition and signalling by TLRs.

Ligand binding to TLRs 2, 4 and 5 induces the recruitment of MyD88 and the downstream activation of NFκB and induction of inflammatory cytokines. TLR4 is also internalized and delivered to the endosome. Recruitment of TRIF leads to activation of late-phase NFκB and also IRF3, which promotes the production of type I interferon. Ligand binding to TLR3 induces recruitment of TRIF and activation of NFκB and IRF3 to promote the production of inflammatory cytokines and type I interferon. TLRs 7 and 9, on the other hand, recruit MyD88 after ligand binding, triggering activation of NFκB and IRF7 to promote the production of inflammatory cytokines and type I interferon. Reviewed in [36].
1.2.2 Negative regulation of TLR signalling

Initiation of TLR signalling leads to the activation of multiple inflammatory pathways that are critical for host defence. However, these pathways must also be tightly regulated, as TLRs have been implicated in the pathogenesis of some autoimmune and chronic inflammatory and infectious diseases [42]. Consequently, TLR signalling is regulated at every level of the pathway. Negative regulation occurs at the level of the TLR receptor itself and the adaptors, as well as at various points along the signalling pathways. These actions are achieved through various mechanisms, including: (1) blocking of ligand binding; (2) downregulation of the receptor; (3) interference with or dissociation of adaptor complexes; (4) degradation of signalling proteins; and (5) transcriptional regulation of components of the TLR signalling cascade and target genes (reviewed in [59] and [42]).

1.3 TLR4

TLR4 recognizes bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria. LPS consists of three distinct components: (1) highly hydrophobic lipid A; (2) hydrophilic polysaccharides that include the core oligosaccharide group and; (3) repeating saccharide subunits, attached to the core oligosaccharide group, known as the O-polysaccharide, O-chain, or O-antigen [60, 61] (Figure 1.5A). Bacteria with full length O-antigen are considered “smooth”, whereas bacteria lacking the O-antigen are considered “rough”. The core oligosaccharide group in Escherichia coli (E. coli) LPS is rich in negatively charged 2-keto-3-deoxyoctulosonic acid (KDO) groups and phosphorylated L-glycerol-D-manno-heptose (Hep) groups [62], which are shielded by the O-antigen in
smooth bacteria. Lipid A consists of a glucosamine backbone acylated by six lipid chains. These hydrophobic fatty acid chains of the lipid A anchor the LPS to the bacterial membrane and the core and O-antigen project out from the membrane (Figure 1.5B). The lipid A portion of LPS binds to TLR4 [63] and is responsible for the toxic effects of LPS [64].

![Diagram of LPS structure](image)

**Figure 1.5. Structure of LPS.**
(A) Schematic of *E. coli* LPS. LPS is composed of three distinct regions: (1) a hydrophilic phosphorylated disaccharide with attached hydrophobic fatty acid chains (lipid A); (2) a core group of oligosaccharides (core); and (3) repeating saccharide units (O-antigen). Lipid A is highly conserved between bacterial strains, while the O-antigen varies greatly from strain to strain. EtN, ethanolamine; Hep, L-glycerol-D-manno-heptose; Gal, galactose; Glu, glucose; KDO 2-keto-3-deoxyoctulosonic acid; NGa, N-acetyl-galactosamine; NGc, N-acetyl-glucosamine; P, phosphate. (B) Bacteria with full-length O-antigen are considered “smooth” (S-LPS) while those lacking most or all of the O-antigen are considered “rough” (R-LPS) or “semi-rough” (SR-LPS). *Figure 1.5B modified from [65] and adapted with permission from SAGE publications, © 2005.*

LPS elicits a powerful inflammatory response and dysregulated responses to LPS are a major contributor to the development of sepsis and septic shock [66]. Septic shock is characterized by the massive release of cytokines and chemokines, the activation of
complement, clotting cascades and leukocytes, and can lead to multiple organ dysfunction syndrome and death [67]. Sepsis represents a considerable burden on the health care system, contributing to longer hospital stays and often leading to death. A 2009 study by the Canadian Institute for Health Information revealed that, in 2008-2009, mortality for all sepsis patients in Canada was 30.5% [68]. This statistic rose to 45.2% for patients diagnosed with severe sepsis, which was defined by the presence of infection and a systemic inflammatory response further complicated by organ dysfunction. The established links between LPS and the development of sepsis and septic shock have thus prompted great interest in studying host responses to LPS and delineating the signalling pathways that are activated by LPS.

In the 1960s and 1970s, strains of mice were identified that showed impaired responses to LPS. In the late 1990s, Poltorak et al. showed that the impaired LPS responses in C3H/HeJ [69] and C57BL/10ScCr [70] mice correlated with defects in the Tlr4 gene [71]. A point mutation in the TIR domain of TLR4 was identified in C3H/HeJ mice [72, 73], while in C57BL/10ScCr mice (renamed C57BL/10ScN in 2004) the entire TLR4 locus is deleted [73, 74]. The critical role of TLR4 in LPS responses was then formally demonstrated in mice with targeted disruption of the Tlr4 gene [72]. Extensive exploration of TLR4-deficient mice and their wild-type counterparts has clearly demonstrated the absolute requirement for TLR4 in numerous responses to E. coli LPS. Indeed, these mice are resistant to the endotoxic shock induced in normal mice by treatment with LPS, yet are more susceptible to infection by gram-negative bacteria [69, 70]. Macrophages deficient in TLR4 fail to activate the NFκB pathway and to produce nitric oxide or cytokines in response to LPS [72]. The lack of functional TLR4 also abrogates the mitogenic effect of LPS on B cells [72]. These
observations led to the prevailing paradigm that signalling through TLR4 is required for all cellular responses to \textit{E. coli} LPS, regardless of the cell type or the biological response considered [75, 76].

1.3.1 TLR4 signalling

As the first TLR identified, the TLR4 signalling pathway is well characterized and is often presented as a model for general TLR signalling due to its extensive study and unique use of both the MyD88 and the TRIF signalling pathways.

TLR4 signalling is initiated by the binding of two MD-2 molecules and two TLR4 molecules to the lipid chains of the lipid A portion of LPS at the plasma membrane [63]. The association of TLR4 and MD-2 occurs prior to LPS binding. When LPS binds the receptor complex, five of the six lipid chains of \textit{E. coli} LPS associate with the hydrophobic pocket of MD-2. The sixth lipid chain is left exposed on the surface of MD-2, where it can associate with another TLR4 molecule. This, in cooperation with the binding of the two phosphate groups on the glucosamine backbone of lipid A to MD-2 and two TLR4 molecules, triggers dimerization of the receptor complex [63]. Additional accessory proteins such as cluster of differentiation 14 (CD14) and LPS-binding protein (LBP) greatly enhance LPS responses, but are not strictly required for TLR4 signal transduction [46, 77]. These proteins are thought to bind LPS and deliver LPS to the TLR4/MD-2 signalling complex [77]. Association of LPS with TLR4/MD-2 prompts the recruitment of the TIR domain-containing intracellular adaptor TIRAP/Mal and, through TIRAP/Mal, MyD88, initiates MyD88-dependent signalling [52] (Figure 1.6).
MyD88 is recruited to TLR4 via TIRAP and subsequently recruits the interleukin-1 receptor associated kinase (IRAK) family members to the signalling complex. Activation of the IRAK proteins results in dissociation with MyD88 and interaction with TNF receptor associate factor 6 (TRAF6), an E3 ubiquitin ligase. TRAF6, together with the ubiquitin conjugating enzymes Ubc13 and Uev1A, catalyzes the addition of polyubiquitin chains on target proteins, including TRAF6 itself. The polyubiquitin chains on TRAF6 serve as a scaffold to recruit the TGF-β activated kinase 1 (TAK1) and IκB kinase (IKK) complexes through their respective ubiquitin-binding proteins: TAK1 binding protein 1 (TAB1) and TAB2 for TAK1 and NFkB essential modulator (NEMO/IKKγ) for the IKK complex (IKKα/IKKβ). The close proximity of TAK1 and the IKK complex facilitates TAK1-mediated phosphorylation and activation of IKKβ. IKKβ-mediated phosphorylation of inhibitor of kappa B (IκB) triggers IκB degradation, facilitating the release of NFkB and its translocation to the nucleus where it induces the expression of pro-inflammatory genes. TAK1 also phosphorylates upstream kinases in the MAPK pathway, leading to activation of p38MAPK, c-Jun N-terminal kinases (JNK) and extracellular signal-regulated kinases (ERK). Downstream effects of the MAPK pathway include activation of transcription factors such as activation protein 1 (AP-1) and modulation of translation. Reviewed in [36].
Following initiation of MyD88 signalling at the plasma membrane, the TLR4 signalling complex then undergoes dynamin-dependent endocytosis and traffics to the endosome, where it recruits the TIR-domain containing adaptors TRAM and TRIF [57, 78]. Signals downstream of TRIF trigger type I interferon production as well as late-phase MAPK and NFκB activation, as TRIF signalling occurs subsequent to MyD88 activation [55, 78] (Figure 1.7). TLR4 signalling is unique in that it requires both MyD88 and TRIF for maximal production of inflammatory cytokines, whereas other TLRs rely on only MyD88 or TRIF [79].
Figure 1.7. TRIF signalling pathway.
TRIF is recruited to the TLR4 signalling complex via TRAM. TRIF recruits TRAF6 through a distinct multi-protein complex that includes the adaptors TNF receptor type 1-associated death domain protein (TRADD) and receptor-interacting protein 1 (RIP1) and the E3 ubiquitin ligase Pellino-1. This complex, including TRAF6, recruits and activates the TAB1/TAB2/TAK1 complex, leading to subsequent activation of the MAPKs, the canonical IKK complex (NEMO/IKKα/IKKβ) and NFkB. TRIF also interacts with TRAF3, which recruits and activates the non-canonical IKK signalling complex composed of TANK-binding kinase 1 (TBK1) and IKKi (IKKe). The TBK1/IKKi complex catalyzes the phosphorylation of IRF3 and its translocation to the nucleus, where it induces expression of type I interferon. Reviewed in [36].

1.4 Other LPS-binding proteins

Although TLR4 is clearly a critical signalling receptor for LPS, there are many other proteins that bind LPS and modulate LPS-induced responses. These include proteins such as CD14 [80-82], scavenger receptors [83-88], the CD11 and CD18 integrins [89-93], heat-shock
proteins [94-98], surfactant proteins [99], the chemokine receptor CXCR4 [95], growth differentiation factor-5 (GDF5) [95], the purinergic receptor P2X7 [100], the seven-span transmembrane receptor CD55 [101], the membrane protein Moeisin [102], potassium (K+) channels [103] and receptor for advanced glycation end products (RAGE) [104], among others. The fact that LPS appears to bind indiscriminately to so many diverse proteins, which each function in distinct ways, has made it challenging to assess their individual contributions to LPS responses. To truly evaluate their specific roles in LPS responses, a system is required where both TLR4 and/or the LPS binding protein in question are absent or non-functional.

1.4.1 CD14

CD14 is a glycoprotein that exists in two forms: a membrane bound, glycosylphosphatidylinositol (GPI)-anchored form and a soluble form. Although CD14 was among the first LPS-binding proteins identified [80], the lack of a transmembrane domain led to questions as to how the GPI-linked protein might mediate LPS responses. LPS-induced secretion of pro-inflammatory cytokines is impaired in the absence of CD14 [80, 105] and CD14-deficient mice are highly resistant to endotoxic shock [81]; however, CD14 is dispensable for some LPS-induced responses [106]. Interestingly, CD14 was reported to associate with the Src family kinase (SFK) Lyn [82] and there is evidence suggesting that ligation of multiple GPI-linked proteins can lead to activation of SFKs co-localized on the inside of the plasma membrane, perhaps through clustering of lipid rafts [82, 107, 108]. Although CD14 is not strictly required for LPS responses, the current understanding is that CD14 acts as a co-receptor for TLR4, binding LPS and facilitating its transfer to the
TLR4/MD-2 signalling complex [77]. CD14 also binds a wide variety of other ligands in addition to LPS, including peptidoglycan [109], lipoteichoic acid, [109, 110] various lipoproteins [111], anionic phospholipids [112] and nucleic acids [113, 114].

1.4.2 Scavenger receptors
Scavenger receptors are transmembrane proteins that bind and uptake a wide variety of polyanionic molecules such as lipoproteins, polyribonucleotides and various bacterial components, including LPS [115-118]. Members of both class A and B of the scavenger receptor family are known to bind and internalize LPS [83, 117, 119]. Class A scavenger receptors also play a role in phagocytosis of bacteria [120-122]. The class B scavenger receptor CD36 was identified in complexes with TLR2/6 [123] and TLR4/6 [124]. CD36 and other class B scavenger receptors were also shown to associate with various SFK members [125, 126]. Finally, mice lacking scavenger receptors are more susceptible to septic shock; they produce more tumor necrosis factor (TNF) and IL-6 [85], but less IL-1β [86] and IL-10 [127], implicating a role for scavenger receptors in cytokine responses after LPS or E. coli challenge.

1.5 p21Ras
The binding of many extracellular ligands to their receptors (including growth factors, hormones, cytokines and chemokines, antigen and extracellular matrix components) results in the activation of members the p21Ras family of small guanosine triphosphatases (GTPases) [128]. The four family members, H-Ras, N-Ras, K-Ras 4A and K-Ras 4B, function as molecular switches and are activated by guanine exchange factors (GEFs) that
catalyze the exchange of guanine diphosphate (GDP; inactive Ras) for guanine triphosphate (GTP; active Ras). Active Ras proteins bind to a wide range of effector proteins that include phosphoinositide 3-kinase (PI3K), GEFs that activate other small GTPases, protein kinases that activate the MAPK pathways and phospholipase C, among others [129]. Ras proteins play important roles in cell proliferation [130-132], differentiation [133-136] and survival [137, 138] and were also shown to have roles in inflammatory functions such as cell adhesion [139, 140], migration [141] and cytokine production [142-145].

LPS induces activation of the MAPK family and transcription factors like NFκB and also stimulates the proliferation of immune cell types such as B cells [146, 147] and T cells [148]. As growth factors can initiate many of these processes via p21Ras, there was interest in determining whether LPS also activates p21Ras. Several studies prior to this thesis directly addressed this question, yet yielded no consensus. One study using the macrophage cell line BAC1.2F5, observed that LPS failed to stimulate activation of p21Ras, although they did note activation of ERK by LPS [149]. Another group, meanwhile, reported that stimulation with LPS led to increased Ras activation in astrocytes [150]. Studies using indirect methods of modulating Ras activity implicated a role for p21Ras in LPS-induced responses by showing that p21Ras activity was required for activation of ERK and expression of inducible nitric oxide synthase, early growth response-1, and TNF in response to LPS [150-153]. These studies modified the activity of Ras through the use of either inhibitors of farnesyl transferases, which block localization of p21Ras to cell membranes, or the expression of dominant-negative asparagine to serine mutants at position 17 (S17N) of p21Ras, which sequester GEFs and prevent activation of p21Ras. Unfortunately, these
approaches may also inhibit other molecules in addition to p21Ras. For example, inhibitors of farnesyl transferases may also inhibit the function of other farnesylated proteins, including other Ras family members. Likewise, the dominant-negative S17N mutants of p21Ras can also inhibit the activation of other members of the Ras family by sequestering GEFs required for the activation of GTPases other than p21Ras [129]. The GEF Son of Sevenless homolog 1 (SOS1), for example, activates not only p21Ras, but also M-Ras, TC21, and the more distantly related Rho family member Rac-1 [154], meaning that S17N mutants of p21Ras could potentially block activation of these other GTPases. Another caveat of these assays is that many were performed several hours after stimulation by LPS and thus may measure not only the direct actions of LPS, but also effects secondary to the LPS-induced secretion of autocrine factors such as TNF or IL-1.

1.6 Specific aims

At the time these studies were initiated, the involvement of p21Ras proteins in the signal transduction pathway of LPS was uncertain; some studies implied their participation while others did not. The initial objective was to resolve the controversy over whether LPS directly activates p21Ras in macrophages. Our surprising observation that LPS-induced activation of p21Ras does not require the canonical LPS signalling receptor TLR4 led us to hypothesize the existence of an additional signalling receptor(s) for LPS in macrophages. Thus, this novel observation prompted our further investigation of: (1) additional LPS-induced, TLR4-independent signals; (2) the contribution of other LPS-binding proteins to TLR4-independent events; (3) the biological role(s) of TLR4-independent signalling; and finally (4) the regulation of p21Ras by LPS and other TLR ligands.
Chapter 2. Materials and methods

All data shown in this thesis are representative of at least three independent experiments.

2.1 Mice

All animal experiments were approved by the University of British Columbia Animal Care Committee. All mice were maintained in a pathogen-free environment.

2.1.1 Source

Wild-type C57BL/6 mice were bred in-house. TLR4-deficient C57BL/10ScNJ (TLR4\(^{-/-}\), stock number 003752) and CD14-deficient B6.129S-Cd14\(^{tm1Frm}\)/J (CD14\(^{-/-}\), stock number 003726) mice were purchased from The Jackson Laboratory and bred in-house. Wild-type C57BL/10J (stock number 000665), wild-type C3H/HeOuJ (stock number 000635), TLR4-signalling-impaired C3H/HeJ (stock number 00659), CD11b-deficient B6.129S4-Itgam\(^{tm1Myd}\)/J (stock number 003991) and CD18-deficient B6.129S7-Itgb2\(^{tm1Bay}\)/J (stock number 002128) mice were purchased from The Jackson Laboratory. SR-AI/II\(^{-/-}\)/CD36\(^{-/-}\) mice [155] were kindly provided by Dr. Urs Steinbrecher. RAGE-deficient mice [156] were generously provided by Dr. Ann Marie Schmidt. MyD88-deficient [157] and TRIF-deficient [55] mice were obtained and used by kind permission of Dr. Shizuo Akira.

2.1.2 Generation of mice deficient for both TLR4 and CD14

Mice lacking both TLR4 and CD14 (TLR4\(^{-/-}\)/CD14\(^{-/-}\)) were generated by crossing C57BL/10ScNJ and B6.129S-Cd14\(^{tm1Frm}\)/J animals. Progeny were screened for TLR4 and
CD14. Presence of TLR4 was assessed using the primer sets listed in Table 2.1. Wild-type littermates were tested to confirm identical responses to C57BL6 and C57BL10/J mice in all assays.

Table 2.1. Primers used for genotyping.

<table>
<thead>
<tr>
<th>Primer Sets</th>
<th>Sequence</th>
<th>Product</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4 (set 1)</td>
<td>sense (5'-GCA AGT TTC TAT ATG CAT TCT C-3')</td>
<td>Wild-type allele: none</td>
<td>Primer sets and PCR conditions are described in Poltorak et al. [74].</td>
</tr>
<tr>
<td></td>
<td>antisense (5'-CCT CCA TTT CCA ATA GGT AG-3')</td>
<td>Mutant allele: 140 bp</td>
<td></td>
</tr>
<tr>
<td>TLR4 (set 2)</td>
<td>sense (5'-TTA GTG TAG GGA CTT CCA CAG-3')</td>
<td>Wild-type allele: 117 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>antisense (5'-GTG GAA ATC ACT TCC TGA TAC-3')</td>
<td>Mutant allele: none</td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>wild-type sense (5'-CCA AGT TTT AGC GCT GCG TAA C-3')</td>
<td>Wild-type allele: 840 bp</td>
<td>Primers and PCR conditions are described in The Jackson Laboratory Genotyping Protocols Database for B6.129S-Cd14&lt;sup&gt;tm1Frm&lt;/sup&gt;/J mice [158].</td>
</tr>
<tr>
<td></td>
<td>mutant sense (5'-CCG CTT CCA TTG CTC AGC GG-3')</td>
<td>Mutant allele: 600 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>antisense (5'-GCC AGC CAA GGA TAC ATA GCC-3').</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: bp, base pairs

2.2 Chemicals and reagents

2.2.1 Cell culture reagents

Dulbecco’s Modified Eagle Media (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 media were purchased from Gibco, as were L-glutamine, sodium pyruvate, nonessential amino acids (NEAA), penicillin and streptomycin. Fetal bovine serum (FBS) and Hank’s Balanced Salt Solution (HBSS) were also purchased from Gibco. 2-Mercaptoethanol (2-ME) was obtained from Fisher and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) was purchased from Sigma-Aldrich.
2.2.2 **Inhibitors**

The inhibitors PP2, LY294002, wortmannin, GM6001, U0126 and SB 203580 were all purchased from Calbiochem. Polymyxin B sulfate was obtained from Sigma-Aldrich.

2.2.3 **Pattern-recognition receptor ligands**

LPS from *E. coli* strain J5 was obtained from Calbiochem. Ultra-pure LPS from *E. coli* K12 and *E. coli* 0111:B4 strains were purchased from InvivoGen, as were lipoteichoic acid from *Staphylococcus aureus*, CpG oligodeoxynucleotides (ODN) 1668 and polyinosinic:polycytidylic acid (poly(I:C)). Synthetic lipid A (compound 506) was purchased from the Peptide Institute. CpG ODN 1826, and intralipid were obtained from Sigma-Aldrich.

2.2.4 **Cytokines and other stimulants**

Colony-stimulating factor 1 (CSF-1), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 were obtained from PeproTech. Phorbol 12-myristate 13-acetate (PMA) was from Sigma-Aldrich.

2.2.5 **Antibodies**

2.2.5.1 **Antibodies used for flow cytometry**

Antibodies used for flow cytometry are listed in Table 2.2.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Catalogue</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>anti-CD11b</td>
<td>M1/70</td>
<td>25-0112-81</td>
<td>eBiosciences</td>
</tr>
<tr>
<td>anti-CD11c</td>
<td>HL3</td>
<td>553802</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>anti-CD16/CD32</td>
<td>2.4G2</td>
<td>553141</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>anti-CD40</td>
<td>3/23</td>
<td>553791</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>anti-CD45</td>
<td>30-F11</td>
<td>557235</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>anti-CD86</td>
<td>GL1</td>
<td>553692</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>anti-F4/80</td>
<td>BM8</td>
<td>n/a</td>
<td>Generated in-house</td>
</tr>
<tr>
<td>anti-Gr-1</td>
<td>RB6-8C5</td>
<td>48-5931-80</td>
<td>eBiosciences</td>
</tr>
<tr>
<td>anti-H-2Kb</td>
<td>AF6-88.5</td>
<td>553570</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>anti-I-Ab</td>
<td>AF6-120.1</td>
<td>553551</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>anti-neutrophil</td>
<td>7/4</td>
<td>ab53453</td>
<td>Abcam</td>
</tr>
</tbody>
</table>

List of antibodies used for flow cytometry in this thesis.

### 2.2.5.2 Antibodies used for immunoblotting and immunoprecipitation

Antibodies used for immunoblotting and immunoprecipitation are listed in Table 2.3 and Table 2.4.
Table 2.3. Non-phospho-specific antibodies used for immunoblotting and immunoprecipitation.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalogue</th>
<th>Source</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-β-actin (clone BA3R)</td>
<td>n/a</td>
<td>Generated in-house</td>
<td>1:50 000 (I.B.)</td>
</tr>
<tr>
<td>anti-Bcl-2 (clone 50E2)</td>
<td>2870</td>
<td>Cell Signaling</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>anti-Caspase-1 (p10)</td>
<td>sc-514-</td>
<td>Santa Cruz</td>
<td>1:500 (I.B.)</td>
</tr>
<tr>
<td>anti-cleaved Caspase-3 (Asp175)</td>
<td>9661</td>
<td>Cell Signaling</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>anti-Cbl</td>
<td>sc-170</td>
<td>Santa Cruz</td>
<td>1:500 (I.B.)</td>
</tr>
<tr>
<td>anti-CrkL</td>
<td>sc-319</td>
<td>Santa Cruz</td>
<td>1:500 (I.B.)</td>
</tr>
<tr>
<td>anti-CSF-1R</td>
<td>sc-692</td>
<td>Santa Cruz</td>
<td>1:500 (I.B.)</td>
</tr>
<tr>
<td>anti-c-IAP2</td>
<td>sc-7944</td>
<td>Santa Cruz</td>
<td>1:500 (I.B.)</td>
</tr>
<tr>
<td>anti-IL-1β</td>
<td>8689</td>
<td>Cell Signaling</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>anti-IL-3/IL-5/GM-CSFR beta chain (βc)</td>
<td>sc-678</td>
<td>Santa Cruz</td>
<td>1:500 (I.B.)</td>
</tr>
<tr>
<td>anti-cleaved PARP (Asp214) (clone 7C9)</td>
<td>9548</td>
<td>Cell Signaling</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>anti-Pyk2</td>
<td>sc-1514</td>
<td>Santa Cruz</td>
<td>1:500 (I.B.)</td>
</tr>
<tr>
<td>anti-Ras (clone RAS10)</td>
<td>05-516</td>
<td>Millipore</td>
<td>1:2000 (I.B.)</td>
</tr>
<tr>
<td>anti-SOS1/2</td>
<td>sc-259</td>
<td>Santa Cruz</td>
<td>1:500 (I.B.)</td>
</tr>
<tr>
<td>anti-Syk</td>
<td>sc-929</td>
<td>Santa Cruz</td>
<td>1:500 (I.B.)</td>
</tr>
<tr>
<td>anti-Vav</td>
<td>sc-8039</td>
<td>Santa Cruz</td>
<td>1:500 (I.B.)</td>
</tr>
</tbody>
</table>

List of non-phospho-specific antibodies used for immunoblotting and immunoprecipitation in this thesis. Antibodies used for immunoprecipitation (I.P.) are noted, as are dilutions used for immunoblotting (I.B.).
Table 2.4. Phospho-specific antibodies used for immunoblotting.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Phospho-site(s)</th>
<th>Catalogue</th>
<th>Source</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-phospho-Akt</td>
<td>Ser473</td>
<td>9271</td>
<td>Cell Signaling</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>anti-phospho-ERK1/2</td>
<td>Thr202/Tyr204</td>
<td>9101</td>
<td>Cell Signaling</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>Anti-phospho-IκBα</td>
<td>Ser32</td>
<td>9241</td>
<td>Cell Signaling</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>anti-phospho-paxillin</td>
<td>Tyr118</td>
<td>2541</td>
<td>Cell Signaling</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>anti-phospho p38 MAP kinase</td>
<td>Thr180/Tyr182</td>
<td>9211</td>
<td>Cell Signaling</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>anti-phospho-MAPKAPK-2</td>
<td>Thr334</td>
<td>3041</td>
<td>Cell Signaling</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>anti-phospho-SHP-2</td>
<td>Tyr542</td>
<td>3751</td>
<td>Cell Signaling</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>anti-phospho-STAT5</td>
<td>Tyr694</td>
<td>9351</td>
<td>Cell Signaling</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>anti-phospho Syk</td>
<td>Tyr525/526</td>
<td>2711</td>
<td>Cell Signaling</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>anti-phosphoserine (clone 4A4)</td>
<td>Ser</td>
<td>05-1000</td>
<td>Millipore</td>
<td>1:1000 (I.B.)</td>
</tr>
<tr>
<td>anti-phosphotyrosine (clone 4G10)</td>
<td>Tyr</td>
<td>005-1050</td>
<td>Millipore</td>
<td>1:1000 (I.B.)</td>
</tr>
</tbody>
</table>

List of phospho-specific antibodies used for immunoblotting in this thesis. Dilutions used for immunoblotting (I.B.) are noted.

2.3 L929 cell conditioned media

L929 cells were cultured in DMEM supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 U/L) and streptomycin (100 μg/L). Cells were grown in tissue culture-treated flasks (BD Biosciences) until confluent and culture media was changed (day 0). Cells were grown for seven days before supernatants were harvested (day 7). Fresh complete culture media was replaced and cells were grown for an additional seven days before supernatants were again harvested (day 14). All supernatants were filtered through 0.2 μm filter bottles (Corning) and equal parts of day 7 and day 14 supernatants were combined for use as L929 cell conditioned media (LCCM). LCCM was used at a 1X concentrate for all experiments.

2.4 Isolation of bone marrow

Mice were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. Both femurs and tibias were removed and bone marrow was flushed out with RPMI 1640 using a
25G5/8 needle. Red blood cells (RBC) were lysed in ammonium-chloride-potassium (ACK) lysing buffer (Gibco) and bone marrow cells were cultured as indicated.

2.5 Generation of bone marrow-derived macrophages

Bone marrow cells were cultured for six to nine days in RPMI 1640 supplemented with FBS (10%), sodium pyruvate (1 mM), penicillin (100 U/L) and streptomycin (100 μg/L) plus LCCM (10%) as a source of CSF-1. Purity of the culture was confirmed by flow cytometry using anti-F4/80 (BM8). Briefly, cells were detached from plastic using Cell Dissociation Buffer (Gibco), washed three times with phosphate-buffered saline (PBS) and then incubated for 30 minutes on ice with anti-CD16/CD32 (FcγRII/III blocker) to prevent non-specific binding of antibodies by Fc receptors. Cells were incubated for 30 minutes on ice with anti-F4/80. After three washes with PBS, data was acquired using FACSCalibur with CellQuest software (BD Biosciences) and analyzed using FlowJo software (Treestar, Inc.).

2.6 Enzyme-linked immunosorbent assay

Bone marrow-derived (BM-derived) macrophages were cultured for six days as described above, detached from the plastic by treatment with trypsin (Gibco), then seeded at $2 \times 10^4$ cells/well in 24-well plates containing 0.5 mL/well RPMI 1640 medium supplemented with FBS (10%) and LCCM (10%). Twenty-four hours later, cells were stimulated by the addition of ultra-pure LPS from *E. coli* K12 (5 μg/mL), synthetic lipid A (1 μg/mL), CpG ODN 1826 (1 μM) or were left untreated. The concentration of TNF-α present in the supernatants after six hours of treatment was assessed using a mouse TNF-α immunoassay kit (R&D Systems).
following the protocol recommended by the manufacturer. Values are graphed as mean ± standard deviation of three replicates.

2.7 B cell blastogenesis assay

Splenocytes were isolated from C57BL/10 or C57BL/10ScN mice and treated with NH$_4$Cl (0.1 M) to eliminate RBC. The remaining cells were cultured at a density of 2 × 10$^6$ cells/mL for 48 hours in RPMI 1640 supplemented with FBS (10%) and 2-ME (100 μM) and with LPS (5 μg/mL), lipid A (1 μg/mL) or CpG ODN 1826 (1 μM) where indicated. Flow cytometry was used to determine cell size (forward scatter) and cell viability, as assessed by exclusion of 7-aminoactinomycin D (7-AAD; Molecular Probes). Cells were washed three times in PBS containing FBS (3%) and sodium azide (0.05%), then incubated with 7-AAD (2 μg/mL) for 20 minutes at room temperature (23°C) in the dark and washed thoroughly. Twenty thousand events were acquired for each sample using FACSCalibur and CellQuest software (BD Biosciences) and analyzed using FlowJo software (Treestar, Inc.).

2.8 Dendritic cell maturation marker assay

Bone marrow cells were cultured for eight days in DMEM supplemented with FBS (10%), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/L), streptomycin (100 μg/L), HEPES (10 mM), NEAA (0.1 mM) and 2-ME (12.5 mM) plus 1.5% GM-CSF containing conditioned media (10X concentrate) derived from GM-CSF-transfected X63-Ag8-plasmacytoma. Dendritic cells were cultured with 5 μg/mL K12 LPS for 18 hours and then washed three times with PBS. Cells were incubated for 30 minutes on ice with anti-CD16/CD32 (FcyRIII/II blocker). Next, cells were stained with anti-CD11c, anti-H-2Kb and
anti-I-Ab to confirm purity and also with anti-CD40 and anti-CD86 to identify maturation markers. All antibodies were incubated with antibody for 30 minutes on ice. Data was acquired using LSR II with FACSDiva software (BD Biosciences) and analyzed using FlowJo software (Treestar, Inc). Mean fluorescent index of CD40 or CD86 surface expression are graphed ± standard deviation of three experiments.

2.9 Macrophage stimulation and lysate preparation

BM-derived macrophages were cultured for seven days before stimulation. Media, FBS and LCCM were replaced on day 6 of culture. Before stimulation on day 7 of culture, the adherent macrophages were washed thoroughly with RPMI 1640 medium supplemented with FBS (5 - 10%) and cultured for one to two hours without LCCM. Where indicated, cells were incubated with designated inhibitors for one hour prior to stimulation and lysis. Where indicated, cells were pretreated with TLR ligands prior to additional stimulation with cytokines or PMA. Also where indicated, LPS was incubated with polymyxin B (50 μg/mL) for 30 minutes at room temperature (23°C) before use. Cells were lysed on ice in a buffer containing Nonidet P-40 (1%), Tris (pH 7.5, 50 mM), NaCl (200 mM), MgCl₂ (5 mM), glycerol (15%), and a mixture of protease inhibitors (Complete Mini, EDTA-free; Roche). Lysates were clarified by centrifugation at 15,000 x g for 10 minutes at 4°C and then stored at -80°C. The protein levels in the samples were quantified using the BCA Protein Assay according to the manufacturer's directions (Pierce).
2.10 Immunoblotting

2.10.1 Images in thesis

Immunoblots displayed in thesis are representative of at least three independent experiments. Bars between lanes in immunoblot images (e.g. Figure 3.3) indicate where irrelevant lanes have been excised for simplicity.

2.10.2 Whole cell lysates

Equalized amounts of protein for each sample (50 μg of lysates) were mixed with 3X sample buffer composed of Tris (pH 6.8, 375mM), glycerol (30%), sodium dodecyl sulfate (SDS; 6%), 2-ME (3%) and bromophenol blue (0.1%) then boiled for five minutes. Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (8-12% separating, 5% stacking). Proteins were transferred to nitrocellulose membranes by wet transfer for 90 minutes at 70 Volts then blocked for one hour in bovine serum albumin (3%) in TBS-T (Tris-buffered saline plus 0.1% Tween-20). The presence of specific proteins was assessed by immunoblotting nitrocellulose membranes with indicated primary antibodies overnight at 4°C. Membranes were washed three times with TBS-T and incubated with secondary antibodies for one hour at room temperature (23°C). Membranes were then washed three times in TBS-T.

For immunoblots analyzed using the enhanced chemiluminescence (ECL) detection system, membranes were incubated with anti-mouse-horseradish peroxidase (HRP) or anti-rabbit-HRP secondary antibodies from DAKO. After washes, membranes were incubated with Western Lightening ECL reagent (PerkinElmer) and imaged on BioMax MR film (KODAK).
using the KODAK M35A X-OMAT Processor. For immunoblots analyzed using the LI-COR Odyssey detection system, membranes were incubated with anti-mouse 800CW or anti-rabbit 680 secondary antibodies from LICOR. After washes, membranes were visualized using the Odyssey Infrared Imaging Systems (LI-COR Biosciences).

2.10.3 Nuclear proteins
BM-derived macrophages were prepared for stimulation, stimulated and lysed as described above in section 2.9, except that lysates were not resolved by centrifugation. Instead, lysates were subjected to sonication using a circulating water bath biorupter (Diagenode) set at 4°C. Lysates were passed through a 20G needle to disrupt DNA fragments and added to 3X sample buffer. The equivalent of 50 μg of lysates for each sample were resolved by SDS-PAGE and immunoblotted as described above.

2.11 Immunoprecipitation and pull-down assays
2.11.1 Assay for activated p21Ras
Activated p21Ras (Ras-GTP) was affinity-precipitated from clarified lysates using Glutathione-Sepharose 4B beads (GE Healthcare) coupled to a recombinant fusion protein of glutathione S-transferase and the Ras-binding domain of Raf-1 (GST-RBD Raf-1), as previously described [128]. Briefly, approximately 20 μg of GST-RBD Raf-1 bound to Glutathione-Sepharose 4B was incubated with 500 μg of clarified lysates for one hour at 4°C to precipitate activated H-Ras, N-Ras, or K-Ras. Beads were washed three times in ice-cold lysis buffer, and boiled in 3X sample buffer. The eluted proteins were resolved by 12%
SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with anti-p21Ras (RAS10). Membranes were analyzed as described in section 2.10.2.

2.11.2 Immunoprecipitation
A total of 500 μg of clarified lysate was incubated with 3 μg of immunoprecipitating antibody for 90 minutes at 4°C before the addition of protein A/G agarose beads (Santa Cruz Biotechnology) for an additional one hour at 4°C. Beads were washed three times in ice-cold lysis buffer, and boiled in 3X sample buffer. The eluted proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with indicated phospho-specific antibodies. Immunoblots were stripped for 30 minutes at 42°C in a buffer containing Tris (pH 6.7, 62.5 mM), SDS (2%) and 2-ME (100 mM) and then washed four times with TBS-T before immunoblotting with immunoprecipitating antibodies. For identification of co-immunoprecipitating proteins, blots were probed with indicated antibodies after stripping off of phospho-specific antibody.

2.11.3 GST-Grb2 pull-down assay
The fusion protein of GST and full-length growth factor receptor-bound protein 2 (Grb2) was described previously [159]. Approximately 20 μg of GST-Grb2 bound to Glutathione-Sepharose 4B (GE Healthcare) was incubated with 500 μg of clarified lysates for one hour at 4°C. Beads were washed three times in ice-cold lysis buffer, and boiled in 3X sample buffer. The eluted proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with indicated antibodies and analyzed as described above.
2.12 WST-1 cell viability assays

BM-derived macrophages were cultured for six days as described above, detached from the plastic by treatment with Cell Dissociation Buffer, then seeded at $1 \times 10^4$ cells/well in 96-well plates containing 0.1 mL/well RPMI 1640 medium supplemented with FBS (10%). Where indicated, LPS and/or LCCM (10%, as a source of CSF-1) were added and cells were cultured for 24 or 48 hours. Cell viability was assessed using water soluble tetrazolium salt 1 (WST-1) reagent (Roche), as per the manufacturer’s directions. Briefly, 10 μl/well of WST-1 reagent was added to wells after culture and incubated in humidified atmosphere (37°C and 5% CO₂) for 90 minutes. Plates were then read on a SpectraMax 190 Absorbance Microplate Reader (Molecular Devices) at 450 nm, subtracting 690 nm background. Optical densities were converted in ‘relative units’ by comparing to a standard curve of absorbance versus cell number, with ‘1’ corresponding to the value obtained with cells cultured in the presence of CSF-1 but not treated with LPS. Values are graphed as mean ± standard deviation of three replicates.

2.13 Assay for assessing changes in macrophage morphology

2.13.1 F-actin staining

Macrophages were cultured for six days as described above, then detached from tissue-culture flasks using Cell Dissociation Buffer (GIBCO) and seeded onto glass chambered slides (NUNC) at approximately $2.95 \times 10^4$ cells per cm². They were then cultured for 20 hours in RPMI 1640 media supplemented with FBS (10%) and LCCM (10%), in the presence or absence of ultra-pure K12 LPS. Cells were fixed in 4% paraformaldehyde for 20 minutes. Cells were then stained for two hours with Alexa Fluor 546-conjugated
phalloidin (Invitrogen) to detect F-actin and washed with PBS. Slides were mounted using Fluoromount-G (SouthernBiotech). Digital images of random fields were captured using a CCD camera (QImaging) mounted on a Zeiss Axioplan 2 microscope and images were analyzed using Openlab software 4.0.4 (Improvision).

2.13.2 Quantification of activated macrophages

Macrophages stained for F-actin were prepared as described above in section 2.13.1, with the following changes: cells were stained with Alexa Fluor 488-conjugated phalloidin (Invitrogen) and chambered slides were mounted using VECTASHIELD mounting media containing DAPI (Vector Labs). Digital images of five random fields at 10X magnification were captured as above. The criteria for scoring resting versus activated phenotype were based on the ratio of cell body to nucleus. Cells that were greater than twice the nuclear diameter in length and equal to or less than twice the nuclear diameter in width were scored as resting (i.e. long and spindle-shaped). Cells that were greater than twice the nuclear diameter in width were scored as activated (i.e. rounded up).

2.14 Wound healing assay

Macrophages were cultured and detached from tissue-culture flasks as described above. 4.5 x 10^5 cells/well in 24-well ImageLock plates (Essen BioScience). Scratches were made with the 24-well Wound Maker tool (Essen BioScience) and wells were washed three times with RPMI 1640. Cells were cultured for 24 hours in RPMI 1640 supplemented with FBS (10%) and LCCM (10%), with or without 5 μg/mL ultra-pure K12 LPS. Images were captured every hour from three separate regions over the 24 hour period using a 20X
objective. The IncuCyte HD system and IncuCyte software (Essen BioScience) were used to capture images and analyze wound confluence.

### 2.15 In vivo leukocyte recruitment

Four age-matched female mice (13 - 20 weeks) were used for each treatment group. Mice were injected with 4 μg per gram body weight (gbw) ultra-pure K12 LPS in HBSS or HBSS alone into the peritoneal cavities. Six hours after injection, peritoneal lavage was performed using 10 μm latex fluorescent beads (Invitrogen) in PBS containing FBS (2%). Cells obtained from lavage fluid were pelleted and incubated for 30 minutes on ice with anti-CD16/CD32 (FcγRII/III blocker) to prevent non-specific binding of antibodies by Fc receptors. Next, cells were stained with anti-CD45, anti-7/4, anti-CD11b, anti-Gr-1 and anti-F4/80. All antibodies were incubated on ice for 30 minutes. Data was acquired using LSRII with FACSDiva software (BD Biosciences) and analyzed using FlowJo software (Treestar, Inc). Cell numbers were calculated relative to the ratio of fluorescent beads retrieved from peritoneal lavage vs. beads injected into the peritoneal cavity at the start of lavage.

### 2.16 Microarray

BM-derived macrophages were cultured for six days as described above, detached from the plastic by treatment with Cell Dissociation Buffer, then seeded at 1 × 10^6 cells/well in 6-well plates containing 2.5 mL/well RPMI 1640 medium supplemented with FBS (10%) and LCCM (10%, as a source of CSF-1). Cells were cultured for six hours in the presence of 5 μg/mL K12 LPS and then harvested using the RNeasy Mini Kit (Qiagen) with Qiashredders (Qiagen), as per the manufacturer’s instructions. RNA was quantified by NanoDrop
Complimentary DNA (cDNA) was prepared from 1 µg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), as per the manufacturer’s instructions. The Affymetrix GeneChip Mouse Gene 1.0 ST Array (Affymetrix) was used to achieve whole-transcript coverage of 28,853 well-annotated genes on duplicate samples. Arrays were run on the Affymetrix platform by skilled technicians at the Centre for Drug Research and Development, University of British Columbia. Analysis was performed using Partek Genomics Suite (Partek Incorporated). Within Partek Genomics Suite, data from the array was normalized and converted to log₂ and ANOVA was performed to calculate average fold difference between LPS treated and untreated normalized signals for both wild-type and TLR4⁻/⁻ macrophages. 966 genes showed changes in gene expression in TLR4⁻/⁻ macrophages following treatment with LPS. Genes with a minimum four-fold increase in gene expression in TLR4⁻/⁻ macrophages following treatment with LPS were selected as genes of interest (p ≤ 0.05, as determined by Partek Genomics Suite). Hierarchical clustering was used to generate a heat map where gene expression was standardized to make the mean zero and the standard deviation one for all genes.

2.17 Quantitative reverse-transcription polymerase chain reaction

cDNA was prepared as described above. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Fermentas). Samples were run in duplicate on 384 well PCR plates. A pPCR mix for each primer set was made from 5 µL SYBR Green Master Mix, 2 µL milliQ water and 1 µL primers (2 µM forward and reverse primers, respectively, see Table 2.5 for sequences);
2 μL cDNA (500 ng/μL) was used for the amplification of each gene. Reactions were carried out in an ABI 7900 real-time PCR machine (Applied Biosystems). Table 2.6 outlines the program used to amplify all genes.

Table 2.6 outlines the program used to amplify all genes.

### Table 2.5. Primers used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Il1a</td>
<td>sense (5′-AGG GCA GAG AGG GAG TCA ACT-3′)</td>
</tr>
<tr>
<td></td>
<td>antisense (5′-AAC TTT GGC CAT CTT GAT TTC TTT-3′)</td>
</tr>
<tr>
<td>Il1b</td>
<td>sense (5′-CAA CCA ACA AGT GAT ATT CTC CAT G-3′)</td>
</tr>
<tr>
<td></td>
<td>antisense (5′-GAT CCA CAC TCT CCA GCT GCA-3′)</td>
</tr>
<tr>
<td>Cxcl3</td>
<td>sense (5′-GGC CCC AGG CTT CAG ATA AT-3′)</td>
</tr>
<tr>
<td></td>
<td>antisense (5′-CCG GCT CAG CT GGA CTT T-3′)</td>
</tr>
<tr>
<td>Irg1</td>
<td>sense (5′-CAG GCT CCC ACC GAC ATA TG-3′)</td>
</tr>
<tr>
<td></td>
<td>antisense (5′-TGT CAT CAA AAT CCA TGG AGT GA-3′)</td>
</tr>
<tr>
<td>Saa3</td>
<td>sense (5′-AAG ATG GGT CCA GTT CAT GAA AG-3′)</td>
</tr>
<tr>
<td></td>
<td>antisense (5′-GAG TAG GCT CGC CAC ATG TCT-3′)</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>antisense (5′-AAA AGG CGC AGT TTA TGT TGT CT-3′)</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>antisense (5′-GGT TCA CTG CAG ACT TGT TCA TG-3′)</td>
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<tr>
<td>Ccl5</td>
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<td></td>
<td>antisense (5′-GAG TAG GCT CGC CAC ATG TCT-3′)</td>
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<tr>
<td></td>
<td>antisense (5′-AGT GCA TGT ACA CCT TGC TGA TC-3′)</td>
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Table 2.6. Thermocycler program used for qRT-PCR.

<table>
<thead>
<tr>
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<th>Time</th>
</tr>
</thead>
<tbody>
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<td></td>
</tr>
<tr>
<td>1. Initial heating</td>
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</tr>
<tr>
<td>Amplification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.* Denaturation</td>
<td>95°C</td>
<td>15 seconds</td>
</tr>
<tr>
<td>3.* Annealing</td>
<td>60°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>4.* Extension</td>
<td>72°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Dissociation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.** Denaturation</td>
<td>95°C</td>
<td>15 seconds</td>
</tr>
<tr>
<td>6.** Annealing</td>
<td>60°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>7.** Denaturation (slow temperature ramp)</td>
<td>95°C</td>
<td>15 seconds</td>
</tr>
</tbody>
</table>

*Steps 2-4 were repeated 40 times. Data was collected at the extension stage (72°C). **Steps 5-7 were added to produce dissociation curves to evaluate formation of primer dimers and non-specific primer binding. Data was collected at dissociation stage between steps 6 and 7 during a slow temperature ramp.

2.17.1 Analysis of qRT-PCR results

The comparative threshold cycle (C_T) method (also known as the \(2^{[\delta\delta]C_T}\) method) [160] was used to analyze our qRT-PCR results, and the stable housekeeping gene RNA polymerase II (RPII) [161] was used as the reference gene for all samples. Mean fold change in gene expression for each treatment was calculated relative to the mean C_T of untreated, wild-type macrophages for each gene evaluated.

2.18 Graphing and statistics

All data presented in graphs were generated using GraphPad Prism (GraphPad Software, Inc.). Where indicated, GraphPad Prism was used to perform unpaired, two-tailed student t-tests or two-way analysis of variance (ANOVA), where indicated. Statistical significance is represented as follows: \(p \leq 0.05\) as (*), \(p \leq 0.01\) as (**) and \(p \leq 0.001\) as (***)
Chapter 3. Lipopolysaccharide-induced activation of p21Ras, Akt and promotion of viability in macrophages does not require Toll-like receptor 4

3.1 Introduction

LPS triggers an intracellular signalling cascade in macrophages that culminates in the release of proinflammatory cytokines and chemokines, contributing to the powerful inflammatory response elicited by gram-negative bacteria. LPS stimulates the activity of a number of key signalling proteins, including SFK members, PI3K, the IKK complex, MAPK family members and transcription factors such as NF-κB, IRFs and AP-1 [162-165]. Many of these responses were shown to depend on the function of TLR4, which was identified as the signalling receptor for LPS 16 years ago [71-73]. Currently, a multimeric complex comprised of TLR4 and accessory proteins such as MD2 and CD14 is acknowledged as the signalling complex for LPS [47, 48, 77]. However, many other proteins were also shown to bind LPS and to modulate LPS-induced responses. These include scavenger receptors [83-88], the CD11 and CD18 integrins [89-93] and RAGE [104], among others. Although these other LPS-binding proteins influence responses to LPS, the current paradigm is that TLR4 is the sole signalling receptor for LPS [75, 76].

Prior to the initiation of this study, the involvement of the small GTPase p21Ras in LPS responses was controversial. Among the best-characterized signal transduction pathways downstream of p21Ras activation is the activation of MAPK family members, particularly activation of ERK1/2 [166]. Thus, there was interest in determining whether LPS also activated MAPK through the activity of p21Ras. However, although several studies directly
examined the activation of p21Ras following LPS stimulation, there was no consensus [149, 150]. Some studies implied a role for p21Ras in LPS-induced cytokine responses [150, 152, 153]; however, these published reports relied on indirect methods of assessing p21Ras involvement, such as farnesyl transferase inhibitors and dominant-negative mutants of p21Ras. In addition, many of these assays were performed hours after LPS treatment and did not take into account any secondary effects of LPS stimulation, such as LPS-induced secretion of autocrine factors. As well, the source of LPS can be problematic as many commercially available preparations of LPS are contaminated with other bacterial components. Isolation of LPS from bacteria requires multiple purification steps to eliminate bacterial phospholipids, proteins, nucleic acids, capsular polysaccharides, peptidoglycan fragments and lipoproteins that are also biologically active [167]. Therefore, rigorous testing is necessary to ensure that the biological effects observed when using LPS are due to the LPS itself and not other contaminating agents in the preparation.

In this chapter, the activation of p21Ras and associated pathways were evaluated in primary macrophages generated *in vitro* from bone marrow progenitors. Surprisingly, we found that ultra-pure LPS activates p21Ras even in the absence of TLR4, the canonical signalling receptor for LPS, and that TLR4-independent activation of p21Ras requires the activities of SFK members and PI3K. Hypothesizing the existence of another signalling receptor for LPS, we established roles for CD14 and the scavenger receptors SR-AI/II and CD36 in TLR4-independent responses. Further investigation of the pathways downstream of TLR4-independent signalling revealed that LPS promotes viability in macrophages through both TLR4-dependent and independent pathways.
3.2 Results

3.2.1 Pure LPS and synthetic lipid A activate p21Ras in primary murine macrophages

To evaluate whether LPS alone can activate p21Ras, it was first necessary to ensure that our preparations of LPS were free of other bacterial contaminants. This was a key concern as ligands for TLR2 and TLR9 had been reported to induce activation of Ras in macrophages [168, 169]. To assess purity, we sought biological assays that would detect low concentration of TLR ligands. It was reported previously that low concentrations of ligands for TLRs 2, 3, 4, 7 and 9 triggered TNF secretion by macrophages [170, 171] and that ligands for TLRs, 2, 4, 7 and 9 induced blastogenesis of B cells [170, 172]. TLRs 2, 4 and 9 were reported to induce maturation of dendritic cells [173, 174], which can be measured by increased expression of the co-stimulatory molecules CD40 and CD86 [175].

Comparing the responses of cells from wild-type mice with cells from mice lacking TLR4 (TLR4−/−) we used three assays to assess purity: production of TNFα from bone marrow (BM)-derived macrophages (Figure 3.1A), blastogenesis of B cells (Figure 3.1B) and upregulation of co-stimulatory markers on BM-derived dendritic cells (Figure 3.1C). In all three assays, our ultra-pure preparations of LPS elicited robust responses in wild-type cells, but did not trigger detectable responses in TLR4−/− cells. The purity of a synthetic lipid A preparation was also confirmed (Figure 3.1A and B). These results indicated that the ultra-pure LPS and synthetic lipid A preparations were not contaminated with biologically significant amounts of other TLR agonists.
Figure 3.1. Ultra-pure preparations of K12 LPS and synthetic lipid A are free of contaminating TLR ligands.

(A) BM-derived macrophages from C57BL/10J wild-type or C57BL/10ScNJ (TLR4−/−) mice were cultured for six hours in the presence of 5 μg/mL ultra-pure LPS from K12 E. coli, 1 μg/mL synthetic lipid A, 1 μM CpG ODN 1826 or were left untreated. Enzyme-linked immunosorbent assay (ELISA) was used to assess the concentration of TNFα in the supernatants. Results are expressed as the mean ± SD of three replicates. (B) Splenocytes from wild-type or TLR4−/− mice were cultured for 48 h in the presence of 5 μg/mL K12 LPS, 1 μg/mL lipid A, 1 μM CpG ODN 1826 or were left untreated. Cell size (forward scatter, FSC) and viability (exclusion of 7-AAD) were assessed by flow cytometry. The percentage of cells in each panel is noted. (C) BM-derived dendritic cells from wild-type or TLR4−/− mice were cultured for 18 hours in the presence of 5 μg/mL ultra-pure K12 LPS or left untreated. Marker expression was assessed by flow cytometry. Graphs show the mean fluorescent intensity (MFI) of CD40 or CD86 surface expression ± SD of three experiments. Two tailed, unpaired t-tests were performed to assess statistical significance of treatment conditions vs. untreated control in both (A) and (B).
Wild-type BM-derived macrophages were then stimulated with these pure preparations of LPS or lipid A. Activation of p21Ras in these cells was assessed by precipitating the active, GTP-loaded form of Ras from cell lysates using a GST-Raf-1 fusion protein and immunoblotting with a monoclonal antibody specific for p21Ras. Macrophages treated with LPS (Figure 3.2A) or lipid A (Figure 3.2B) rapidly increased the levels of activated p21Ras, within three minutes of stimulation. The swift kinetics of this activation led us to conclude that this is a direct effect of LPS. Pretreating the LPS (Figure 3.2A) or lipid A (Figure 3.2B) with polymyxin B, which specifically binds and neutralizes lipid A [176], completely abrogated the activation of p21Ras induced by these preparations. Taken together, these data confirm that LPS alone can activate p21Ras in primary murine macrophages.

**Figure 3.2. Pure LPS and lipid A activate p21Ras.**
Solutions of 50 μg/mL polymyxin B (PBM) were incubated for 30 minutes with or without 5 μg/mL ultra-pure K12 LPS or 10 ng/mL CSF-1 (A) or 1 μg/mL lipid A (B) and then used to stimulate BM-derived macrophages from C57BL/6 wild-type mice for three minutes. Activation of p21Ras was assessed by pull-down assay using GST-Raf1 and immunoblotting for p21Ras.

To evaluate whether different types of LPS can also activate p21Ras, we compared the activation of p21Ras in macrophages stimulated with ultra-pure LPS preparations from *E. coli* that produce LPS without an O-antigen (“rough” LPS, our K12 preparation) to *E. coli* that produce LPS with an O-antigen (“smooth” B4 LPS). We found that smooth LPS only very weakly induced activation of p21Ras, when compared to rough LPS (Figure 3.3A). As
the O-antigen on smooth LPS would contribute to the overall molecular weight of the LPS, we could not rule out the possibility that the diminished effect of smooth LPS was due to a lower overall concentration of lipid A in equivalent molecular weights of smooth and rough LPS. Increasing the dose of smooth LPS, however, was not sufficient to increase this weak activation of p21Ras (data not shown). Treatment with either rough or smooth LPS, however, led to strong phosphorylation of Akt (also known as protein kinase B, PKB) in macrophages, indicating that the smooth LPS preparation was indeed biologically active (Figure 3.3B). Consistently over many different preparations of LPS from various species of rough and smooth *E. coli*, stimulation with rough LPS robustly activated p21Ras and smooth LPS did not (data not shown).

![Figure 3.3](image)

**Figure 3.3. Rough, not smooth, LPS strongly activates p21Ras.** BM-derived macrophages from C57BL/6 wild-type mice were stimulated with 5 μg/mL smooth ultra-pure B4 or rough ultra-pure K12 LPS for indicated times. (A) Pull-down assay for activated Ras, immunoblotted for p21Ras. (B) The same lysates were immunoblotted for phospho-Akt (Ser473) to confirm activity of LPS preparations. Equal loading was confirmed (not shown).
3.2.2 LPS-induced activation of p21Ras occurs in absence of TLR4 and depends on the activity of SFKs and PI3K

While evaluating the purity of our LPS preparations, we made a surprising observation: TLR4−/− macrophages robustly activate p21Ras following five minutes of stimulation with LPS or lipid A (Figure 3.4A). To rule out a strain-specific effect in our mice, we tested another mouse strain defective in the Tlr4 gene, C3H/HeJ [71]. We observed TLR4-independent activation of p21Ras in this strain as well (Figure 3.4B). Having confirmed the purity of our preparations using multiple sensitive assays, we concluded that LPS-induced activation of p21Ras can occur independently of TLR4, although we could not rule out a role for TLR4-dependent activation of p21Ras.

A closer examination of the kinetics of p21Ras activation revealed that p21Ras was activated more rapidly in wild-type macrophages than in TLR4−/− macrophages. After one minute of stimulation with LPS, wild-type macrophages showed a strong activation of p21Ras, whereas p21Ras was only weakly activated in TLR4−/− macrophages at this time point. Following five minutes of LPS stimulation, both wild-type and TLR4−/− macrophages showed strong activation of p21Ras (Figure 3.4C). These observations led us to conclude that there is another, novel signalling receptor for LPS that is capable of inducing p21Ras activation without TLR4 and that, in wild-type cells, TLR4 may also be capable of activating p21Ras or cooperating with the TLR4-independent pathway to activate p21Ras, at least at very early time points (i.e. following one minute of LPS stimulation).
The concentration of LPS used to stimulate activation of p21Ras was chosen based on our previous work showing that 5 μg/mL of conventional (not ultra-pure) LPS yielded the strongest activation of p21Ras following three to five minutes of LPS treatment [177]. We confirmed, using our ultra-pure preparation of LPS, that both wild-type and TLR4−/− macrophages activate optimal levels of p21Ras when treated with 5 μg/mL of LPS from *E. coli* for five minutes (*Figure 3.4D*).
Figure 3.4. Activation of p21Ras by LPS or lipid A does not require TLR4.

BM-derived macrophages from C57BL/10J wild-type and TLR4+/− (A, C & D) or C3H/HeOuJ wild-type and C3H/HeJ (B) mice were treated as follows. (A & B) Synthetic lipid A (1 μg/mL) or ultra-pure K12 LPS (5 μg/mL) for five minutes. (C) 5 μg/mL ultra-pure K12 LPS for one or five minutes, as indicated. (D) Indicated dose of ultra-pure K12 LPS for five minutes. Lysates were subjected to pull-down assay for activated Ras and immunoblotted for p21Ras.

Our group showed previously that, in wild-type macrophages, maximal activation of p21Ras in response to LPS required the activities of SFKs and PI3K [177]. These observations led us to investigate whether TLR4-independent p21Ras activation by LPS also required the activities of these kinases. We found that PP2, a SFK inhibitor, reduced the activation of
p21Ras induced by LPS in both wild-type and TLR4−/− macrophages (Figure 3.5A). Likewise, the PI3K inhibitor LY294002 also reduced LPS-induced p21Ras activation to similar levels in both wild-type and TLR4−/− cells (Figure 3.5B). We concluded that maximal TLR4-independent activation of p21Ras by LPS requires the activities of SFKs and PI3K and that either (1) any signals from TLR4 which contribute to p21Ras activation in wild-type also require the activities of these kinases or, (2) the activation of p21Ras observed following five minutes of LPS stimulation is predominantly signaled via the TLR4-independent pathway.

Figure 3.5. Activation of p21Ras by LPS depends on the activity of SFKs and PI3K. BM-derived macrophages from C57BL/10J wild-type and TLR4−/− mice were treated for one hour with DMSO, 5 μM PP2 (SFK inhibitor) (A), or 5 μM LY294002 (PI3K inhibitor) (B) and then stimulated for three minutes with 5 μg/mL J5 LPS. Lysates were subjected to pull-down assay for activated Ras and immunoblotted for p21Ras.

As PI3K activity is required for maximal p21Ras activation induced through the TLR4-independent pathway, we next examined LPS-induced phosphorylation of Akt, an effector of PI3K. We found that LPS induces serine-phosphorylation of Akt to similar levels in both wild-type and TLR4−/− macrophages (Figure 3.6A). In contrast, the activation of ERK and p38MAPK triggered by LPS was observed only in wild-type macrophages (Figure 3.6B). Taken together, these observations indicate that LPS-induced activation of p21Ras and Akt
occur in a TLR4-independent manner, whereas activation of ERK and p38MAPK is strictly dependent on TLR4.

Figure 3.6. Activation of Akt by LPS does not require TLR4.
BM-derived macrophages from C57BL/10J wild-type and TLR4−/− mice were treated with 5 μg/mL ultra-pure K12 LPS for indicated times. (A) Lysates immunoblotted for phospho-Akt (Ser473). (B) The same lysates immunoblotted for phospho-ERK (Thr202/Tyr204) and phospho-p38MAPK (Thr180/Tyr182). Equal loading was confirmed (not shown).

3.2.3 CD14 and class A and B scavenger receptors contribute to LPS-induced p21Ras activation
Our observations that TLR4−/− macrophages activated the PI3K and p21Ras pathways led us to hypothesize the existence of a novel, alternate signalling receptor for LPS that signals independently of TLR4. We sought to identify the receptor(s) mediating LPS-induced activation of p21Ras by investigating cell surface proteins known to bind LPS and modulate cellular responses to LPS. We were able to rule out a requirement for the CD11b (Figure
3.7A), CD18 (Figure 3.7B) and RAGE (Figure 3.7C) in LPS-induced p21Ras activation by using macrophages derived from transgenic mice lacking the indicated protein. Using macrophages derived from MyD88 and TRIF knockout mice, we were also able to rule out other TLRs as candidates for the alternate LPS signalling receptor (Figure 3.7D & E).

Figure 3.7. CD11b, CD18, RAGE and other TLRs do not contribute to LPS-induced activation of p21Ras.
BM-derived macrophages from C57BL/6 wild-type and CD11b⁻/⁻ (A), CD18⁻/⁻ (B), RAGE⁻/⁻ (C), MyD88⁻/⁻ (D) or TRIF⁻/⁻ (E) mice were treated with 5 μg/mL ultra-pure K12 LPS for five minutes. Lysates were subjected to pull-down assay for activated Ras and immunoblotted for p21Ras.

CD14 was first identified as the LPS signalling receptor [80]. However, unresolved questions as to how CD14, a GPI-linked protein, could link signals from outside the plasma membrane to the cytosol made CD14 an unlikely signalling molecule. Thus, with the discovery of TLR4, CD14 was relegated to the role of carrier protein that transferred LPS to the TLR4 signalling complex. Still, evidence that CD14 and other GPI-linked proteins associate with SFKs [82, 107] and that ligation of GPI-linked proteins can lead to activation of SFKs [108] prompted us to investigate the contribution of CD14 to p21Ras activation. We
found that CD14−/− macrophages only weakly activated p21Ras after one to five minutes of LPS stimulation (Figure 3.8A). Our laboratory previously observed p21Ras activation in macrophages following treatment with the lipid emulsion intralipid (unpublished observations), thus macrophages were also treated with intralipid to rule out intrinsic defects in p21Ras activation in CD14−/− macrophages. Both wild-type and CD14−/− macrophages showed approximately equivalent levels of activated p21Ras in response to intralipid stimulation, indicating that CD14−/− macrophages do not have a defect in p21Ras activation. Our observations following one minute of LPS stimulation in wild-type and TLR4−/− macrophages led us to hypothesize that TLR4 contributes to early p21Ras activation, and the diminished p21Ras activation following one and three minutes of LPS stimulation on CD14−/− macrophage support this interpretation, as CD14 has a well-established role as a co-receptor for TLR4. After five minutes of LPS treatment, however, CD14−/− macrophages still show only weak activation of p21Ras. This suggests that CD14 contributes to the TLR4-independent activation of p21Ras, as at this time point we observed approximately equivalent levels of activated p21Ras in wild-type and TLR4−/− macrophages stimulated with LPS. Examination of Akt in wild-type macrophages revealed phosphorylation of Akt as rapidly as five minutes after stimulation with LPS, and this activity was sustained through to 30 minutes of LPS treatment. CD14−/− macrophages, however, did not phosphorylate Akt until 30 minutes of LPS stimulation (Figure 3.8B). This is in contrast to TLR4−/− cells, which showed no defect in Akt phosphorylation at the same kinetic (i.e. 10 and 15 minutes, Figure 3.6C). These results suggest that CD14 contributes to early Akt phosphorylation, but is not fully responsible for TLR4-independent Akt phosphorylation observed at 30 minutes following LPS treatment.
Figure 3.8. CD14 contributes to LPS-induced activation of p21Ras and Akt. BM-derived macrophages from C57BL/6 wild-type and CD14$^{-/-}$ mice were treated with 5 μg/mL ultra-pure K12 LPS for indicated times or with 150 μg/mL intralipid for 30 minutes. (A) Lysates were subjected to pull-down assay for activated Ras and immunoblotted for p21Ras. (B) Lysates were immunoblotted for phospho-Akt (Ser473). Equal loading was confirmed (not shown).

Scavenger receptors bind to a variety of molecules such as lipoproteins, polyribonucleotides and various bacterial components, including LPS [115-118]. Various members of the family are implicated in modulating cytokine responses after LPS or E. coli challenge [85, 86, 127]. Several class A members play a role in LPS and E. coli phagocytosis [120-122] while class B members were reported to activate p21Ras following ligand engagement [178]. We found that macrophages lacking both class A and B family members (Scavenger receptor class A type I/II-CD36 double knockout macrophages, SR-AI/II$^{-/-}$/CD36$^{-/-}$) showed reduced levels of activated p21Ras as compared to wild-type macrophages stimulated with LPS, whereas both activated similar levels of p21Ras in response to CSF-1 (Figure 3.9A). The low levels of p21Ras induced by five minutes of treatment with LPS in SR-AI/II$^{-/-}$/CD36$^{-/-}$ macrophages could be contributed by additional scavenger receptor family members and/or the actions of
CD14 and TLR4 in these cells. The data in Figure 3.8 and Figure 3.9, taken together with our observations in wild-type and TLR4-deficient macrophages (Figure 3.4C), suggest that activation of p21Ras following one minute of LPS stimulation depends predominantly on the activities of TLR4 and CD14, whereas activation of p21Ras after five minutes of LPS treatment depends more upon the activities of class A and B scavenger receptors and CD14. Akt phosphorylation was also examined and was found to be equivalent in both wild-type and SR-AI/II^{−/−}/CD36^{−/−} macrophages (Figure 3.9B), indicating that these scavenger receptors do not play a role in LPS-independent activation of Akt.

**Figure 3.9.** SR-AI/II and CD36 are required for maximal activation of p21Ras induced by LPS. BM-derived macrophages from C57BL/6 wild-type and SR-AI/II^{−/−}/CD36^{−/−} mice were treated with 5 μg/mL ultra-pure K12 LPS for indicated times or with 10 ng/mL recombinant CSF-1 for three minutes. (A) Lysates were subjected to pull-down assay for activated Ras and immunoblotted for p21Ras. (B) Lysates were immunoblotted for phospho-Akt (Ser473). Equal loading was confirmed (not shown).
3.2.4 TLR4 signalling limits the duration of p21Ras activation

To further characterize the TLR4-independent activation of p21Ras, we examined extended kinetics of LPS stimulation in wild-type and TLR4−/− macrophages. We found that wild-type macrophages show maximal levels of activated p21Ras by five minutes stimulation with LPS and then rapidly drop below background levels by 10 minutes stimulation. TLR4−/− macrophages, on the other hand, sustained high levels of activated p21Ras for at least 30 minutes following LPS stimulation (Figure 3.10A). We concluded that signals downstream of TLR4 led to inhibition of p21Ras. We next examined macrophages lacking the TLR adaptors MyD88 or TRIF to identify the pathway through which TLR4 exerts this inhibition of p21Ras. We found that TRIF−/− macrophages behaved similarly to wild-type macrophages, inhibiting p21Ras activation by 10 minutes of LPS stimulation (Figure 3.10B), indicating that TRIF is not involved in the TLR4-dependent inhibition of p21Ras. MyD88−/− macrophages, however, showed a delayed kinetic of p21Ras inhibition (Figure 3.10C). At 10 and 15 minutes following LPS treatment, MyD88−/− cells showed sustained activation of p21Ras. By 30 minutes of LPS stimulation, however, MyD88−/− macrophages show inhibition of p21Ras. Examination of the role of CD14 in this pathway revealed, like MyD88−/− macrophages, activation of p21Ras at 10 minutes following LPS treatment in CD14−/− macrophages that was inhibited by 30 minutes (Figure 3.10D). Thus, CD14 and MyD88 potentiate TLR4-driven inhibition of p21Ras, but are not strictly required. Further investigation of this inhibitory pathway is included in chapter 5 of this thesis.
3.2.5 LPS promotes viability via TLR4-dependent and independent pathways

LPS has been shown in some cases to induce death in macrophages, and to promote their survival in others [179-183]. Our observation that the PI3K and p21Ras pathways are activated in response to LPS, but that p21Ras signalling is attenuated rapidly in wild-type
macrophages, prompted us to investigate the relative contributions of the LPS-induced, TLR4-dependent and -independent pathways to macrophage survival. Using the WST-1 metabolic assay as a measure of cell viability, we found that wild-type macrophages cultured concurrently with their growth factor CSF-1 and LPS for 24 hours showed decreasing viability as LPS concentrations increased. TLR4\(^{-/-}\) macrophages cultured under the same conditions, however, showed increased viability as LPS concentrations increased (Figure 3.11A).

This pattern was even more apparent after 48 hours co-culture with LPS, with higher doses (5 \(\mu\)g/mL) of LPS exerting a marked positive effect on TLR4\(^{-/-}\) macrophage viability (Figure 3.11B). We noted that TLR4\(^{-/-}\) macrophages treated with higher doses of LPS had higher values on the WST-1 assay than resting macrophages cultured only in CSF-1. We attributed this effect to macrophage death in the resting, CSF-1 only, cultures of wild-type and TLR4\(^{-/-}\) macrophages that we took as our baseline for this experiment. During the course of our macrophage experiments, we consistently observed that confluent dishes or wells of mature macrophages (as cultured in these particular experiments) required daily refreshment of CSF-1 for optimal viability. Thus, we concluded that LPS exerts a protective effect on TLR4\(^{-/-}\) macrophages that is opposed by TLR4 in wild-type macrophages. One caveat of WST-1 assay is that it measures the reduction of tetrazolium salts by dehydrogenase enzymes and, as such, is actually a measure of the net metabolic activity of the cell (reviewed in [184]). Thus, it was possible that the elevated assay values in wild-type and TLR4\(^{-/-}\) macrophages treated with LPS might reflect increased metabolism rather than increased viability. While we cannot completely rule out the possibility that LPS might affect macrophage metabolism,
we were able to confirm by phase microscopy the presence of greater numbers of live cells in LPS-treated wells that correlated strongly with the results of the WST-1 assays (data not shown).

We were interested in examining the protective effect of LPS more closely in a model where cell death could potentially be prevented. As macrophages depend on CSF-1 for their survival [185], we used withdrawal of CSF-1 as a method of inducing macrophage death. Within 24 hours following cytokine removal, we observed a large decrease in macrophage viability. Adding lower doses of LPS (200 ng/mL) to macrophages at the time of CSF-1 withdrawal substantially increased the survival of wild-type macrophages, whereas the effect on TLR4−/− macrophages was minimal (Figure 3.11C). In contrast, culturing macrophages with higher doses of LPS (5 μg/mL) greatly increased the survival of TLR4−/− macrophages following CSF-1 withdrawal. Higher doses of LPS also promoted viability in wild-type macrophages, although the degree of protection was somewhat reduced as compared to that induced by low doses of LPS. We found similar results in macrophages cultured with LPS for 48 hours following LPS removal (Figure 3.11D). From these observations of macrophages undergoing cell death induced by growth factor removal, we concluded that, at lower doses, LPS induces macrophage survival through a pathway that requires TLR4, although we cannot rule out a role for the TLR4-independent pathway aiding the TLR4-dependent pathway. At higher doses, however, LPS promotes viability primarily through a TLR4-independent pathway.
Figure 3.11. LPS promotes viability via TLR4-dependent and independent pathways.

WST-1 assays were performed to assess cell viability of BM-derived macrophages from C57BL/6 wild-type and TLR4−/− mice. (A & B) Macrophages were cultured with LCCM as a source of CSF-1 and concurrently stimulated with indicated doses of ultra-pure K12 LPS for 24 (A) or 48 hours (B). (C & D) Macrophages were cultured in absence of LCCM and concurrently stimulated with indicated doses of ultra-pure K12 LPS for 24 (C) or 48 (D) hours. Reference values of 1.0, based on macrophages cultured in the presence of LPS alone for 24 (A & C) or 48 (B & D) hours, were calculated from a standard curve of number of cells vs. absorbance. All experimental samples were compared to these reference values and are graphed ± SD. Two-way ANOVA followed by Bonferroni post-hoc test was used to assess statistical significance of treatment conditions vs. untreated control.
Having established roles for scavenger receptors and CD14 in the p21Ras pathway, we next assessed these proteins for a role in LPS-induced viability. We found that CD14–/– macrophages co-cultured with CSF-1 and LPS showed a dose-independent protective effect at both 24 and 48 hours (Figure 3.12A & B), as compared to wild-type macrophages. This could indicate that CD14 plays a role in the TLR4-dependent block of the TLR4-independent promotion of viability we observed in wild-type and TLR4–/– macrophages under the same conditions (Figure 3.11A & B). When CD14–/– macrophages were cultured with LPS after CSF-1 removal, we observed LPS-induced protective effects on these cells that were greater than that in wild-type macrophages under the same conditions (Figure 3.12C & D). Surprisingly, even low doses of LPS were sufficient to induce a protective effect in CD14–/– macrophages. Taken together, these experiments indicate that CD14 negatively impacts LPS-induced pro-survival signalling. CD14 may play a critical part in the negative impact TLR4 has on LPS-induced viability, however, in this scenario its role in TLR4-dependent promotion of viability at low doses would be negligible. We also concluded that CD14 does not play a role in the TLR4-independent viability pathway as we observed an increase in the viability of CD14–/– macrophages compared to wild-type cells.
Figure 3.12. CD14 participates in the TLR4-dependent reduction of LPS-induced viability.
WST-1 assays were performed to assess cell viability of BM-derived macrophages from C57BL/6 wild-type and CD14⁻/⁻ mice. (A & B) Macrophages were cultured with LCCM as a source of CSF-1 and concurrently stimulated with indicated doses of ultra-pure K12 LPS for 24 (A) or 48 (B) hours. (C & D) Macrophages were cultured in absence of CSF-1 and concurrently stimulated with indicated doses of ultra-pure K12 LPS for 24 (C) or 48 (D) hours. Reference values of 1.0, based on macrophages cultured in the presence of LCCM alone, were calculated from a standard curve of number of cells vs. absorbance. All experimental samples were compared to these reference values and are graphed ± SD. Two-way ANOVA followed by Bonferroni post-hoc test was used to assess statistical significance of treatment conditions vs. untreated control.
WST-1 assays performed on SR-AI/II⁺/CD36⁻ macrophages showed responses similar to wild-type cells (data not shown), indicating that LPS does not enhance macrophage viability through scavenger receptors. We also generated mice lacking both TLR4 and CD14 for experiments detailed in Chapter 4 of this thesis (see section 4.2.2). Macrophages generated from these mice were also evaluated in WST-1 assays and showed similar response to TLR4⁻/⁻ mice (data not shown), indicating the TLR4-independent pathway was still functional in these cells. Thus, the identity of the receptor(s) contributing to TLR4-independent viability is still unknown.

From our WST-1 cell viability assay results, we hypothesized that LPS treatment was inhibiting apoptosis of the cells cultured without CSF-1. To confirm this, we examined apoptosis markers. Twenty-four hours after CSF-1 withdrawal, we observed cleavage of poly ADP-ribose polymerase (PARP) [186] and cleavage of cysteine-dependent aspartate specific protease 3 (caspase 3) [187] in wild-type and TLR4⁻/⁻ macrophages (Figure 3.13A). We found that treatment of macrophages with 5 μg/mL LPS blocked cleavage of these proteins in both wild-type and TLR4⁻/⁻ macrophages. Taken together, these data confirm that LPS indeed blocks apoptosis in a TLR4-independent manner. Consistent with the cleavage of PARP and caspase 3, we also observed a decrease in the levels of B cell lymphoma 2 (Bcl2), a pro-survival protein [187], in both wild-type and TLR4⁻/⁻ macrophages 24 hours after CSF-1 removal (Figure 3.13B). We also observed reduced levels of Bcl2 in wild-type, but not TLR4⁻/⁻ macrophages treated with LPS in the presence of CSF-1, indicating that LPS was triggering pro-apoptotic events in wild-type cells. Consistent with this, we observed another pro-apoptotic response, cleavage of cellular inhibitor of apoptosis 2 (cIAP2) [188],
in wild-type macrophages treated with LPS (Figure 3.13C). In summary, although these pro-apoptotic signals are transduced via TLR4, globally, the response to LPS appears to be pro-survival, indicating a balance between TLR4-dependent pro-death and TLR4-independent pro-life signals in macrophages.

Figure 3.13. LPS induces pro-survival and pro-apoptotic signals.
BM-derived macrophages from C57BL/6 wild-type and TLR4−/− mice were cultured for 24 hours with and without 5 μg/mL ultra-pure K12 LPS and LCCM as a source of CSF-1, as indicated. Lysates were immunoblotted with antibodies against (A) cleaved PARP (top), cleaved caspase 3 (bottom), (B) Bcl2 and (C) cIAP2. Equal loading was confirmed (not shown).
3.3 Discussion

The experiments detailed here demonstrate unequivocally that pure LPS from a rough strain of *E. coli* activates p21Ras without the requirement for TLR4. Obtaining LPS preparations free of contaminating bacterial products was of paramount importance to not only ascertain whether LPS alone could activate p21Ras, but also to validate our surprising observation that p21Ras activation does not require TLR4. This was particularly important, as other TLR ligands were shown to activate p21Ras [168, 169]. We used four different assays in three different primary cell types to rigorously test our LPS preparations. ELISA assays for TNFα secretion in macrophages, blastogenesis of B cells, and upregulation of maturation markers on dendritic cells all ruled out the presence of low levels of contaminating TLRs. The ability of polymyxin B to completely abrogate p21Ras activation by our ultra-pure preparations of K12 LPS and lipid A further confirmed purity, thus validating our observations that LPS activates PI3K and p21Ras to promote viability in absence of TLR4. Although infection with bacteria is unlikely to result in the exposure of macrophages to LPS in the absence of other bacterial PAMPs, it is clear that LPS responses are a critical component to the development of sepsis and septic shock. It is important to note that the TLR4-independent responses described here also occur in wild-type cells, although some of these responses are limited in scope. Our observations indicate the existence of other signalling receptors for LPS that can function independently of TLR4 and that likely co-operate with TLR4 to elicit the overall macrophage response to LPS.

There has been some controversy over the ability of LPS to activate p21Ras. Several groups reported failure of LPS to stimulate activation of Ras [149] or stimulate very weak
activation of Ras [189] in macrophages; however, these studies used LPS from a smooth strain of *E. coli* (0111:B4) which we found to only very weakly activate p21Ras as compared to LPS from a rough strain of *E. coli* (K12). This demonstrates that different types of LPS are not equivalent in their ability to induce p21Ras. Our observation that p21Ras activation occurs independently of TLR4 may further explain why there are so many inconsistencies in reports of LPS-induced p21Ras activation. We show here that scavenger receptors and CD14 are important components of the LPS/p21Ras signalling axis. Studies examining Ras activation in cell types or cell lines lacking adequate expression of these, and other signalling molecules important in these pathways, would also have failed to observe Ras activation in response to LPS.

Smooth LPS has three distinct components: (1) the highly hydrophobic lipid A from which extends the hydrophilic polysaccharides that include (2) the core oligosaccharide group and (3) the attached, repeating saccharide subunits (O-polysaccharide, O-chain, O-antigen) [60, 61], whereas rough LPS lacks the hydrophilic O-antigen. Consequently, the negative charges on the core oligosaccharide group are exposed in rough LPS, resulting in greater negative charge in rough LPS as compared to smooth LPS. In addition, the absence of the O-antigen increases the hydrophobicity of rough LPS [190], increasing its aggregation status and altering other physical properties. As the lipid A portion of LPS is conserved between smooth and rough *E. coli* LPS, we hypothesize that the difference in the ability of these different types of LPS to activate p21Ras may be due to the charge and/or aggregation status of these molecules and their ability to interact with and initiate signalling through CD14 and the scavenger receptors.
Although we show here that the SFK/PI3K/Ras pathway occurs via a TLR4-independent pathway, we also observed a strict requirement for TLR4 in LPS-induced activation of p38MAPK and ERK. That p38MAPK activation is not part of the SFK/PI3K/p21Ras pathway is not surprising, as our group previously demonstrated that treatment with SFK inhibitors does not affect activation of p38MAPK by LPS [177]. LPS-induced activation of ERK, on the other hand, is more complex. Several groups, including ours, reported that the LPS-induced Ras and ERK pathways are uncoupled in macrophages [149, 177]. Our group also showed, however, that inhibition of SFKs blocks activation of ERK in response to LPS [177], raising the possibility that either TLR4 on its own may activate SFKs and subsequently ERK, or that integration of signals via the TLR4-dependent and independent pathways are required for ERK activation.

Previous studies demonstrated the requirement for both ERK and p38MAPK activity for LPS-induced cytokine expression [191]. Work by others demonstrated that expression of oncogenic p21Ras is sufficient to induce expression of proinflammatory cytokines and chemokines including IL-6 and IL-8 [142-144] and that constitutively active Raf-1, an effector of p21Ras, can augment the TNF promoter activity induced by LPS [145]. Other groups showed that dominant-negative Ras and inhibitors of Ras and Raf-1 block LPS-induced activation TNF by blocking the TNF promoter [145]. Taken together, these studies suggest that p21Ras, ERK and p38MAPK all play important roles in cytokine expression. We found that TLR4 was absolutely required for ERK and p38MAPK activation and also for TNF-α, IL-6 and GM-CSF secretion stimulated by LPS (IL-6 and GM-CSF data preliminary, not
shown). However, LPS-induced activation of SFK/PI3K/p21Ras occurs independently of TLR4. As noted above, we found previously that SFK activity was required for ERK activation by LPS [177]. Taken together, it is not unlikely that the TLR4-dependent and independent pathway must work together to activate all the elements – SFK, p21Ras, ERK and p38MAPK – required to induce cytokine expression in response to LPS.

The contributions of CD14 and the scavenger receptors SR-AI/II and CD36 to TLR4-independent, LPS-induced pathways are intricate. At very early time points, activation of p21Ras appears to depend predominantly on the activities of TLR4 and CD14, as activation of p21Ras by LPS is stronger in wild-type macrophages at these time points than in TLR4- or CD14-deficient cells. Activation of p21Ras at a median time point, after three minutes of LPS treatment, appears to depend more upon the activities of class A and B scavenger receptors and CD14 than TLR4. Both scavenger receptor-deficient and CD14-deficient macrophages show impaired activation of p21Ras following three minutes of LPS treatment, while LPS-induced activation of p21Ras at this time point is comparable in TLR4-deficient cells and wild-type cells. There are reports that CD14 clustering and that scavenger receptor ligation can lead to SFK activation [108, 192], suggesting a possible mechanism by which these receptors contribute to p21Ras activation. As we found that SFKs and PI3K are upstream of p21Ras activation induced by LPS, one might expect that p21Ras activation would correlate with Akt activation. Indeed, Akt phosphorylation induced by LPS is also TLR4-independent, however, scavenger receptor-deficient macrophages do not show defects in Akt activation induced by LPS even though p21Ras activation is diminished. CD14, on the other hand, does contribute to Akt activation but is not strictly required. The
participation of CD14 in the TLR4-independent activation of p21Ras and Akt clearly demonstrates a role for CD14 in addition to its function as a co-receptor for TLR4. CD14 also contributes to TLR4-dependent signalling in this pathway, evident by the impairment of the TLR4-dependent dampening of p21Ras activation in CD14-deficient macrophages. Despite reports that clustering of CD14 and other GPI-linked proteins may be sufficient to induce activation of SFKs, we cannot rule out that the role of CD14 in TLR4-independent responses may be to help load LPS onto other LPS-signalling receptor(s), similar to its role in the TLR3 and TLR4 pathways [96, 114, 193]. The fact that lack of CD14 delays, but does not completely abrogate, Akt phosphorylation supports the hypothesis that CD14 acts as a co-receptor for receptors other than TLRs. Based on these observations, it is tempting to speculate the existence of another signalling receptor for LPS-induced PI3K that may also contribute to SFK and p21Ras activation.

Our observation that Akt is activated via a TLR4-independent pathway is consistent with the requirement for PI3K upstream of p21Ras. The activation of p21Ras and Akt likely contributes to the LPS-induced, TLR4-independent promotion of macrophage viability. Studies examining the effects of altering the levels of Ras activity demonstrated a role for Ras in macrophage viability [137, 138]. Compared to wild-type macrophages under the same conditions, macrophages with low levels of Ras activity (generated from transgenic mice overexpressing suppressors of Ras activation) exhibited decreased survival following CSF-1 withdrawal [137]. Other studies found that expression of a constitutively active mutant of p21Ras in stem/progenitor cells promotes differentiation into macrophages that survive and grow in the absence of CSF-1 [138], indicating that activated p21Ras can
substitute for growth factors in maintaining the survival of macrophages. Taken together, these findings indicate that Ras activity not only promotes macrophage viability, but may also be required for their survival. PI3K also plays a role in cell survival, in part through Akt-dependent inhibition of pro-apoptotic molecules [194, 195]. Thus, the TLR4-independent activation of p21Ras and/or PI3K likely contribute to the LPS-induced pro-survival effects seen in both wild-type and TLR4 \(^{-/-}\) macrophages. Although we show here that scavenger receptors contribute to p21Ras activation induced by LPS, we did not observe a role for the scavenger receptors in LPS-induced viability. Our observations that CD14-deficient macrophages display an increase in LPS-induced viability led us to conclude that CD14 contributes more to the TLR4-dependent pro-death pathway and that its role in the TLR4-independent pro-survival pathway is negligible. Thus, the receptor(s) contributing to the TLR4-independent, LPS-induced promotion of viability in macrophages still remains to be identified.

We found that using higher doses of LPS (5 μg/mL) yielded greater effects on TLR4-deficient macrophages than lower doses of LPS, particularly in viability assays. This same dose was used in our p21Ras activation assays, as lower doses of ultra-pure K12 LPS did not elicit as strong an activation of p21Ras in wild-type [177] or TLR4-deficient macrophages (Figure 3.4D). The reliance on higher concentrations of LPS to trigger responses in TLR4-deficient cells could be indicative of several scenarios. First, the receptors involved in mediating responses to LPS in the absence of TLR4 may be of lower affinity for LPS than TLR4. Second, higher concentration of LPS may be necessary to ensure the formation of micelles or aggregates of LPS that function to cluster LPS-binding
proteins on the surface of macrophages. Both of these scenarios will be discussed further in Chapter 4, in context with the data presented in that chapter as well.

It is important to note that the responses triggered by LPS in TLR4-deficient macrophages also occur in wild-type cells: activation of p21Ras and Akt, and LPS-induced promotion of viability. In wild-type cells, however, the extent of p21Ras activation is diminished, as is the protective effect of higher doses of LPS on macrophages undergoing apoptosis. Together, these data indicate that the TLR4-dependent pathway influences the TLR4-independent pathway. There has been a great deal of controversy over the role of LPS in macrophage survival. LPS was shown in some cases to induce macrophage death and in others to promote macrophage survival [179-183, 196, 197]. Here, we show that LPS induces TLR4-dependent pro-death signals and TLR4-independent pro-life signals, but that overall response to LPS in wild-type cells appears to be pro-survival, indicating integration of the two pathways. Macrophages derived from different tissues can differ greatly in their gene expression and in their biological responses [10]. Therefore, it is likely that the relative expression levels of TLR4, CD14, the scavenger receptors and additional, non-TLR4, signalling receptor(s) for LPS may vary between populations of macrophages. The experiments detailed in this chapter indicate that the TLR4-dependent and -independent pathways each influence the other and likely co-operate to direct the overall response of the macrophage following encounter with LPS.
Chapter 4. Lipopolysaccharide stimulates Src family kinase-dependent tyrosine phosphorylation, induces cytoskeletal rearrangement and modulates leukocyte trafficking in the absence of Toll-like receptor 4

4.1 Introduction

Protein phosphorylation is a key component of many signalling pathways. The addition or removal of a phosphate group to serine, threonine or tyrosine residues can alter the conformation of a protein and subsequently affect its enzymatic activity, subcellular localization or ability to form interactions with other proteins (reviewed in [198]). Protein kinases catalyze the addition of phosphate groups to these residues while protein phosphatases facilitate the removal of phosphate groups from proteins.

LPS treatment triggers the activation of a wide variety of kinases in macrophages. For example, serine and serine/threonine kinases upstream of the IKK complexes and MAPK family contribute to the activation of transcription factors such as NFκB, IRFs and AP-1 [162]. LPS also activates various tyrosine kinases, including several SFK members [163]. The activities of SFKs are critically important for many functional responses of cells, including cytoskeletal assembly and organization, cell-cell contact, cell-matrix adhesion, induction of DNA synthesis, cell survival and cellular proliferation (reviewed in [199] and [200]).

As noted above, the activities of SFKs contribute to the regulation of actin cytoskeleton dynamics. Modulation of cytoskeleton components mediates the formation of structures
such as lamellipodia, filopodia and focal adhesions, which impact cell morphology, adhesion or motility [201]. These processes are critical for many cellular functions during immune responses, including the recruitment of leukocytes to sites of infection [202]. The migration of leukocytes to target tissues where they can initiate effector functions is an essential component to the resolution of infection.

In Chapter 3 of this thesis, we evaluated the activation of p21Ras by ultra-pure LPS. We found that LPS-induced activation of p21Ras depends on the activities of SFKs and PI3K but does not require TLR4. From this, we hypothesized that tyrosine phosphorylation of SFK targets occurs in the absence of TLR4. We show here that, indeed, LPS triggers SFK-dependent tyrosine phosphorylation without the requirement for TLR4 and that CD14 plays a role in the phosphorylation of some these proteins. We then generated mice lacking both TLR4 and CD14 in order to further investigate the role of CD14 in TLR4-independent responses in vitro and in vivo.

4.2 Results

4.2.1 Pure LPS induces tyrosine phosphorylation of SFK targets in absence of TLR4
To investigate the role of SFKs in LPS-induced tyrosine phosphorylation, we generated BM-derived macrophages from wild-type mice and treated them with ultra-pure LPS for five minutes. We found that LPS stimulation rapidly induced tyrosine phosphorylation of proteins of approximately 120, 100, 70 and 40 kDa (Figure 4.1A). We observed that phosphorylation of the 120, 100 and 70 kDa bands by LPS was inhibited in the presence of PP2, a SFK inhibitor. The phosphorylation of the approximately 40 kDa band, however, was not
impaired by PP2, indicating that the effect of PP2 was specific to its role as a SFK inhibitor and not due to general toxicity. As we previously observed that TLR4-independent activation of p21Ras required the activities of SFKs and PI3K (Figure 3.5), we also examined macrophages deficient in TLR4 to see if these SFK-dependent tyrosine phosphorylation events occurred in absence of TLR4. Indeed, proteins of the same molecular weight – 120, 100 and 70 kDa – were also phosphorylated following LPS treatment in TLR4-deficient mice (Figure 4.1B). In contrast, tyrosine phosphorylation of the approximately 40 kDa band induced by LPS remained dependent on TLR4.

While investigating the role of TLR4 in p21Ras activation (detailed in Chapter 3), we identified a role for CD14 in TLR4-independent signalling. Studies by others indicated that CD14 and other GPI-linked proteins associate with SFKs [82, 107] and that ligation of GPI-linked proteins can lead to activation of SFKs [108]. These data prompted us to investigate the contribution of CD14 to the LPS-induced, SFK-dependent, TLR4-independent tyrosine phosphorylation events. We found that tyrosine phosphorylation of the 120 and 70 kDa proteins was greatly impaired in CD14-deficient macrophages following five minutes of LPS stimulation, whereas LPS-induced tyrosine phosphorylation of the 100 kDa band was not affected by lack of CD14 (Figure 4.1C). We concluded from this experiment that CD14 plays a role in the phosphorylation of the 120 and 70 kDa proteins but does not contribute to the phosphorylation of the 100 kDa band.
Figure 4.1. LPS-induced tyrosine phosphorylation of some SFK targets does not require TLR4.

BM-derived macrophages from indicated mice were stimulated with 5 μg/ml ultra-pure LPS from K12 *E. coli* for five minutes, as indicated. Lysates were immunoblotted with anti-phosphotyrosine antibody 4G10. (A) BM-derived macrophages from C57BL/6 wild-type mice were treated with DMSO or 5 μM PP2 (SFK inhibitor) for one hour prior to stimulation with LPS. (B) BM-derived macrophages were from C57BL/10J wild-type or TLR4−/− mice. (C) BM-derived macrophages were from C57BL/6 wild-type or CD14−/− mice. Arrowheads indicate proteins of interest. Equal loading was confirmed (not shown).

To better understand the role that the TLR4-independent pathway plays in LPS responses, we sought the identity of the various SFK targets that were phosphorylated in both wild-type and TLR4-deficient macrophages. By immunoprecipitating various SFK targets and blotting with anti-phosphotyrosine, we observed tyrosine phosphorylation of the adaptor protein Cbl (120 kDa; Figure 4.2A), the hematopoietic-specific focal adhesion protein proline-rich tyrosine kinase (Pyk2) (120 kDa; Figure 4.2B), the hematopoietic-specific guanine exchange factor Vav (100 kDa; Figure 4.2C) and the cytoplasmic tyrosine kinase spleen tyrosine kinase (Syk) (72 kDa; Figure 4.2D) in both wild-type and TLR4−/− macrophages following five minutes of LPS treatment, although Vav phosphorylation was weak in both wild-type and TLR-deficient macrophages.
Figure 4.2. Identification of proteins targeted by SFK for tyrosine phosphorylation via TLR4-independent pathway.

BM-derived macrophages from C57BL/6 wild-type or TLR4−/− mice were treated with 5 μg/mL ultra-pure K12 LPS for indicated times. Lysates were immunoprecipitated (IPed) with indicated antibodies and immunoblotted with anti-phosphotyrosine antibody 4G10. Immunoblots were stripped and re-probed with immunoprecipitating antibody to confirm equal immunoprecipitation (IP).

Closer scrutiny of later time points revealed that LPS signalling via TLR4 limits the duration of tyrosine phosphorylation on some of the proteins we identified downstream of the TLR4-independent pathway. We found that tyrosine phosphorylation of Cbl and Syk reached approximately equivalent levels by five minutes of LPS stimulation in wild-type and TLR4−/− macrophages. However, by 10 minutes these proteins were no longer phosphorylated in wild-type macrophages, whereas they showed sustained phosphorylation in TLR4−/− macrophages (Figure 4.2A and D). Tyrosine phosphorylation of Pyk2, on the other hand, was still present following ten minutes of LPS stimulation (Figure 4.2B).
Upon further examination of the kinetics of phosphorylation of Cbl, we noted the co-immunoprecipitation of a protein of approximately 40 kDa, which we identified as the adaptor protein Crk-like protein (CrkL) (**Figure 4.3A**). We observed that LPS-induced tyrosine phosphorylation of Cbl and its interaction with phosphorylated CrkL extended beyond 30 minutes in TLR4−/− macrophages, whereas it was limited to five minutes duration in wild-type macrophages. We noted earlier that CD14 was required for maximal tyrosine phosphorylation of proteins of approximately 120 kDa (**Figure 4.1C**), therefore we also evaluated the contribution of CD14 to LPS-induced Cbl phosphorylation. We confirmed the requirement of CD14 for maximal phosphorylation of Cbl after three to five minutes of LPS stimulation. However, levels of Cbl phosphorylation continued to increase in CD14−/− macrophages after this time point and were prolonged as compared to wild-type macrophages (**Figure 4.3B**). Thus, CD14 appears to participate in both the TLR4-independent phosphorylation of Cbl (observed within one to five minutes after LPS stimulation in wild-type and TLR4-deficient macrophages) and the TLR4-dependent inhibition of Cbl phosphorylation (observed by 10 minutes following LPS treatment in wild-type macrophages).

Syk phosphorylation kinetics differed from that of Cbl. While Syk phosphorylation following ten minutes of LPS treatment was inhibited in wild-type macrophages and sustained in TLR4−/− macrophages (**Figure 4.3C**), it was not as long-lived as Cbl phosphorylation in TLR4−/− macrophages (**Figure 4.3A**). By 15 minutes after LPS stimulation, Syk phosphorylation started to decline in TLR4−/− macrophages as well (**Figure 4.4C**).
We also examined MyD88-deficient macrophages to see whether the TLR4-dependent inhibitory pathway operated via the intracellular adaptor MyD88. Indeed, we found that tyrosine phosphorylation of the 120 kDa and 70 kDa bands was still present after 10 and 15 minutes of LPS treatment in MyD88⁻⁻ macrophages (Figure 4.3D). We also observed, however, that the inhibition of the 120 kDa protein, Cbl, was present by 30 minutes of LPS stimulation, indicating that although MyD88 contributes to the TLR4-dependent loss in tyrosine phosphorylation, it is not strictly required.
Figure 4.3. CD14 and MyD88 contribute to TLR4-dependent modulation of LPS-induced tyrosine phosphorylation.

BM-derived macrophages from C57BL/6 wild-type, TLR4<sup>−/−</sup> (A & C), CD14<sup>−/−</sup> (B) or MyD88<sup>−/−</sup> (D) mice were treated with 5 μg/mL ultra-pure K12 LPS for indicated times. (A & B) Lysates were IPed with anti-Cbl antibodies and immunoblotted with anti-phosphotyrosine antibody 4G10. Immunoblot in (A) was also re-probed with CrkL to confirm identity of the co-IPed band. (C) Lysates were immunoblotted for phospho-Syk (Tyr525/526). (D) Lysates were immunoblotted with anti-phosphotyrosine antibody 4G10. Arrowheads indicate proteins of interest. Equal loading was confirmed (not shown).
We also observed LPS-induced tyrosine phosphorylation that strictly required TLR4. In wild-type macrophages, we saw phosphorylation of the scaffolding protein paxillin (Figure 4.4A) following ten minutes of LPS stimulation. Tyrosine phosphorylation of the phosphatase SH2 domain-containing phosphatase-2 (SHP-2) was also observed in wild-type macrophages after ten minutes of LPS treatment (Figure 4.4B). Both paxillin and SHP-2 were not phosphorylated in TLR4\(^{-}\) macrophages at the time points examined.

![Figure 4.4. TLR4 is required for phosphorylation of paxillin and SHP-2.](image)

BM-derived macrophages from C57BL/6 wild-type mice or TLR4\(^{-}\) mice were treated with 5 \(\mu\)g/mL ultra-pure K12 LPS for indicated times. Lysates were immunoblotted for phospho-paxillin (Tyr118) (A) or phospho-SHP-2 (Tyr542) (B), then stripped and re-probed with anti-actin to confirm equal loading.

4.2.2 LPS-induced morphological changes do not require TLR4

We observed TLR4-independent tyrosine phosphorylation induced by LPS on several SFK targets (Cbl, CrkL, Pyk2, Vav and Syk) that elsewhere were reported to mediate cytoskeletal reorganization [203-208]. As cytoskeletal reorganization can promote events such as cell spreading, adhesion and motility [202], we next investigated whether LPS could induce morphological changes in macrophages lacking TLR4. Untreated cells, regardless of their genetic background, displayed a long, spindle-like phenotype (Figure 4.5A, left
panels). We found that overnight treatment with LPS stimulated obvious cytoskeletal arrangements in wild-type macrophages at all LPS doses tested, inducing cell spreading and the appearance of actin-rich structures such as filopodia, lamellipodia and actin ruffles (Figure 4.5A, top row; examples of these actin-rich structures are indicated by open arrows, closed arrows and arrowheads, respectively). In TLR4−/− macrophages, we observed these same morphological changes following 5 μg/mL of LPS treatment, the same concentration of LPS used to induce tyrosine phosphorylation in wild-type and TLR4−/− macrophages. At lower doses of LPS, however, the majority of TLR4−/− macrophages retained a long spindle-like phenotype similar to untreated macrophages (Figure 4.5A, second row from the top and Figure 4.5B).

Our observation that CD14 contributes to some of the LPS-induced, TLR4-independent phosphorylation on tyrosine residues (Figure 4.1C and Figure 4.3B) prompted us to investigate CD14-deficient macrophages to evaluate whether CD14 might contribute to macrophage flattening in absence of TLR4. CD14−/− macrophages displayed a phenotype similar to that of wild-type cells, where LPS induced cytoskeletal changes at all LPS doses tested (Figure 4.5A, second row from the bottom). However, CD14−/− macrophages express TLR4, which mediates LPS-induced morphological changes at low doses of LPS (Figure 4.5A, second row from the top and Figure 4.5B). Thus, we generated mice lacking both TLR4 and CD14 to evaluate the contribution of CD14 to LPS-induced responses in absence of TLR4. Intriguingly, macrophages lacking both TLR4 and CD14 remained completely unresponsive to LPS, even following high doses of LPS (Figure 4.5A, bottom row and Figure 4.5B). This was not due to an inherent defect in these macrophages, as treatment
with the synthetic TLR3 agonist poly(I:C) was sufficient to induce an activated phenotype in TLR4−/−/CD14−/− macrophages (Figure 4.5C). From these experiments, we concluded that either TLR4 or CD14 is required for LPS-induced morphological changes in macrophages. While TLR4 supports LPS-induced morphological changes at a range of LPS doses, CD14 can substitute for TLR4 in eliciting LPS-induced cytoskeletal changes only in conditions where there are higher concentrations of LPS.
Figure 4.5. LPS-induced morphological changes do not require TLR4.
BM-derived macrophages from C57BL/6x10 wild-type, TLR4<sup>−/−</sup>, CD14<sup>−/−</sup> or TLR4<sup>−/−</sup>/CD14<sup>−/−</sup> mice were stimulated with indicated doses of ultra-pure K12 LPS (A) or 25 µg/mL poly(I:C) (C) for 20 hours and then fixed. Fixed cells were stained with fluorophore-labelled phalloidin. (A) Images were captured using a 40X objective. Scale bars represent 50 µm. Examples of filopodia (open arrows), lamellipodia (closed arrows) and actin ruffles (arrowheads) are indicated in wild-type macrophages treated with LPS (top row). (B & C) Five random fields were captured using a 10X objective and macrophages were scored for resting vs. activated phenotypes. Results are expressed as mean ± SD. Two-way ANOVA followed by Bonferroni post-hoc test was used to assess statistical significance of treatment conditions vs. untreated control in (B). Two tailed, unpaired t-tests were performed to assess statistical significance of treatment conditions vs. untreated control in (C).
4.2.3 LPS-induced alteration of migration in wound-healing assays requires TLR4

Another cytoskeletal-dependent process, macrophage motility, was also investigated. Using a simple scratch/wound-healing assay to evaluate macrophage migration, we examined the effect of concurrent LPS treatment on the migration of macrophages into the wounded area. We found that untreated macrophages, cultured in the presence of their growth factor CSF-1, filled in the wounded area at a steady rate. Wild-type macrophages treated with LPS showed little difference in migration rate, as compared to untreated cells, for the first six to eight hours after wounding. After this, however, wild-type macrophages stopped migrating and cell density within the wound did not change for the remainder of the assay (24 hours post-wounding) (Figure 4.6A). TLR4-deficient macrophages treated with LPS did not show the same behavior. TLR4\(^{-/-}\) macrophages treated with LPS migrated to fill the wounds at a similar rate to untreated macrophages (Figure 4.6B). CD14-deficient cells displayed an intermediate phenotype. LPS-treatment caused a delay in the rate at which CD14\(^{+-}\) macrophages filled in the wound, but did not cause a halt in migration as in wild-type cells treated with LPS (Figure 4.6C). The delay in migration of CD14\(^{+-}\) macrophages treated with LPS likely reflects the contribution of CD14 to the TLR4-dependent inhibition of macrophage migration induced by LPS in wild-type cells. LPS-treated TLR4\(^{-/-}\)/CD14\(^{-/-}\) macrophages showed similar responses to LPS-treated TLR4\(^{-/-}\) macrophages and untreated macrophages (Figure 4.6D).
Figure 4.6. LPS alters macrophage migration in wound-healing assays via TLR4.
Confluent wells of BM-derived macrophages from C57BL/6x10 wild-type (A), TLR4\(^{-/-}\) (B), CD14\(^{-/-}\) (C) or TLR4\(^{-/-}\)/CD14\(^{-/-}\) (D) mice were subjected to an *in vitro* scratch assay. Macrophages were cultured with 5 \(\mu\)g/mL ultra-pure K12 LPS after wounding. Migration was quantified by evaluating wound density every hour following wounding using the Incucyte system to assess cell density within the wound. Results are expressed as mean ± SD. Two-way ANOVA was performed on samples to assess statistical significance of treatment conditions vs. control. Wild-type and CD14\(^{-/-}\) macrophages treated with LPS filled wounds significantly slower than untreated controls (\(p<0.0001\) and \(p<0.0021\), respectively). There was no significant difference between treated and untreated macrophages derived from either TLR4\(^{-/-}\) or TLR4\(^{-/-}\)/CD14\(^{-/-}\) mice.

4.2.4 LPS modulates leukocyte distribution in absence of TLR4

*In vivo*, cytoskeletal reorganization facilitates leukocyte recruitment to areas of infection [202]. Another group reported that intraperitoneal (i.p.) injection of LPS into mice lacking CD14 or TLR4 elicited an influx of neutrophils into the peritoneal cavities much earlier than that of wild-type mice. They observed neutrophil recruitment six hours after LPS injection in TLR4- or CD14-deficient animals and 24 hours after LPS injection in wild-type animals [209]. In this study, however, LPS preparations were not tested to ensure that they were not...
contaminated with other TLR agonists and bacterial products. As we could not rule out that
the effect observed by Haziot et al. was due to bacterial contaminants and not to LPS alone,
we examined peritoneal leukocyte populations following i.p. injection of our ultra-pure LPS
into wild-type, TLR4<sup>−/−</sup>, CD14<sup>−/−</sup> and TLR4<sup>−/−</sup>/CD14<sup>−/−</sup> mice. We found that, indeed, ultra-pure
LPS elicited a pronounced influx of 7/4<sup>+</sup>, Gr-1<sup>+</sup> neutrophils into the peritoneal fluid of TLR4<sup>−/−</sup>
and CD14<sup>−/−</sup> mice six hours after LPS injection, but not in wild-type mice at this time point
(Figure 4.7A).

We recovered a significantly greater number of neutrophils from the peritoneal fluid of LPS-
treated TLR4<sup>−/−</sup> animals than from CD14<sup>−/−</sup> animals, suggesting either: (1) CD14 could be at
least partly responsible for the early influx of neutrophils in TLR4<sup>−/−</sup> animals, or (2) CD14 is
an important component in the TLR4-dependent delay in neutrophil recruitment to the
peritoneal cavity observed by Haziot et al. To resolve this, we also examined mice lacking
both TLR4 and CD14. TLR4<sup>−/−</sup>/CD14<sup>−/−</sup> mice also showed recruitment of neutrophils six hours
following LPS injection, but at lower numbers than those recovered from TLR4<sup>−/−</sup> animals.
This led us to conclude that although CD14 may contribute to the influx of neutrophils into
the peritoneal cavity at six hours following LPS injection, there is at least one other receptor
that, in the absence of TLR4, promotes the early entry of neutrophils to the peritoneal cavity.

In addition to early neutrophil recruitment, we observed an influx of 7/4<sup>+</sup>, Gr-1<sup>int</sup> monocytes,
into the peritoneal cavity of TLR4<sup>−/−</sup> animals, but not wild-type animals, six hours after LPS
administration (Figure 4.7B). In contrast to our observations for neutrophil recruitment, we
did not observe an influx of monocytes into the peritoneal fluid of CD14<sup>−/−</sup> animals treated
with LPS. TLR4\textsuperscript{-/-}/CD14\textsuperscript{-/-} animals retained the ability to recruit monocytes to the peritoneal cavity after six hours, indicating that CD14 is not involved in the recruitment of monocytes to the peritoneal cavity six hours after local LPS injection.

We also found a decrease in the number of F4/80\textsuperscript{+}, CD11b\textsuperscript{+} macrophages recovered from the peritoneal fluid of wild-type mice six hours after LPS treatment (Figure 4.7C). TLR4\textsuperscript{-/-}, CD14\textsuperscript{-/-} and TLR4\textsuperscript{-/-}/CD14\textsuperscript{-/-} mice also showed a decrease in the number of macrophages that were recovered from peritoneal fluid after LPS treatment. However, although both TLR4\textsuperscript{-/-} and TLR4\textsuperscript{-/-}/CD14\textsuperscript{-/-} mice showed a significant decrease in the number of macrophages in the peritoneal fluid after LPS treatment, they did not have the same magnitude of reduction as wild-type mice treated with LPS. These observations led us to conclude that TLR4, but not CD14, contributes to the reduction in macrophages in peritoneal fluid after LPS treatment. However, this pathway is predominantly TLR4-independent, as TLR4\textsuperscript{-/-} mice still display this phenotype. Taken together, our observations of peritoneal leukocytes in mice treated with LPS indicate that TLR4\textsuperscript{-/-} mice are responsive to LPS and that there are other LPS binding receptors aside from TLR4 and CD14 that mediate LPS responses \textit{in vivo} in absence of TLR4.
C57BL/6x10 wild-type, TLR4<sup>−/−</sup>, CD14<sup>−/−</sup> or TLR4<sup>−/−</sup>/CD14<sup>−/−</sup> mice were injected with 4 μg/gbw ultrapure K12 LPS or HBSS as mock treatment (n=4 for all treatment groups). Six hours later, peritoneal cavities were flushed with PBS and peritoneal fluids were analyzed for cellular content using flow cytometry. Results are expressed as mean ± SD. A two tailed, unpaired t-test was performed to assess statistical significance of treatment conditions vs. control and to compare treated macrophages from different mouse strains.

**Figure 4.7.** LPS modulates peritoneal leukocyte distribution in absence of TLR4.

4.3 Discussion

The results detailed in this chapter demonstrate that pure LPS stimulates tyrosine phosphorylation of multiple target proteins in primary macrophages through the activity of SFKs and that TLR4 is not required to mediate these effects. We found that, *in vitro*, LPS
induces cell spreading and actin cytoskeleton rearrangement of macrophages in the absence of TLR4. Furthermore, we also found that LPS modulates leukocyte responses in TLR4-deficient animals in vivo, demonstrating the functional significance of the TLR4-independent pathway. Importantly, many of the LPS-induced responses that we observed in TLR4-deficient macrophages (tyrosine phosphorylation, changes in actin dynamics in vitro and macrophage responses in vivo) also occur in wild-type cells and wild-type animals. Together, these findings support the hypothesis that there are additional signalling receptors for LPS that can function independently of TLR4. As well, the data shown here demonstrates that CD14, in addition to its function as a co-receptor for TLR4, also participates in TLR4-independent responses. In the presence of high doses of LPS, CD14 can substitute for the absence of TLR4 to trigger LPS-induced cell spreading and actin cytoskeleton dynamics. Whether CD14 alone is capable of inducing these changes, or whether CD14 functions as a co-receptor for another LPS signalling receptor, remains to be determined.

Our observation that LPS induces SFK-dependent tyrosine phosphorylation of multiple proteins further supports our hypothesis that LPS mediates multiple signalling events in absence of TLR4. Furthermore, identification of the proteins phosphorylated in response to LPS in TLR4-deficient macrophages provided insight to the function of the TLR4-independent pathway. Previous studies demonstrated that inhibitors of SFKs or PI3-kinase can block LPS-induced adhesion and macrophage spreading [210]. Activation of SFKs leads to the phosphorylation of numerous proteins with well-known roles in reorganization of the cytoskeleton and other actin-dependent processes. These proteins include Pyk2, Vav,
Cbl, CrkL and Syk, all of which we observed phosphorylated in a TLR4-independent manner. SFK-dependent phosphorylation of Pyk2 and Syk is implicated in actin cytoskeleton rearrangements that include cell spreading, adhesion and formation of lamellipodia [203]. Vav, once tyrosine phosphorylated, is an activator of the Rho family of small GTPases that regulates actin cytoskeleton dynamics [211, 212]. Cbl interacts with SFKs, Pyk2 and Vav, and was shown to be critical for macrophage flattening and migration [204, 213]. Overexpression of CrkL is sufficient to induce cell flattening and formation of ruffles [214]. Finally, Syk is a non-receptor tyrosine kinase that interacts with SFKs [215], Pyk2 [216], Vav [217], Cbl [218] and CrkL [215] and was shown to phosphorylate microtubules [219] and contribute to cell spreading [207]. The well-documented roles in cytoskeletal organization of these proteins hinted that the TLR4-independent pathway might affect LPS-induced changes in the actin cytoskeleton. Indeed, we observed LPS-induced cell spreading and formation of actin ruffles, lamellipodia and filopodia in TLR4-deficient macrophages.

In addition to our observation that LPS stimulated tyrosine phosphorylation of multiple proteins in both wild-type and TLR4-deficient macrophages, we also observed that LPS-induced phosphorylation of Cbl and Syk was prolonged in TLR4-deficient macrophages, as compared to wild-type macrophages. This demonstrated that signals via TLR4 act to curtail the TLR4-independent phosphorylation, indicating that the TLR4-dependent and TLR4-independent pathways do intersect. One possibility is that TLR4-dependent signalling may be responsible for negatively regulating the activity of upstream signals, anywhere from the receptor through to the kinases responsible for the TLR4-independent phosphorylation.
cascades. Another possibility for the prolonged phosphorylation observed in TLR4-deficient cells could be the activation of phosphatases in wild-type macrophages via a TLR4-dependent pathway. Our observation that tyrosine phosphorylation of the phosphatase SHP-2 occurs at the same time point as attenuation of Cbl and Syk phosphorylation prompts speculation for a role for SHP-2 in the TLR4-dependent termination of phosphorylation seen in wild-type cells. Cbl and SHP-2 were observed to strongly interact in T cells [220], and roles for SHP-2 in negatively regulating LPS responses have been described by others [221, 222]. However, the TLR4-dependent phosphorylation of SHP-2 could be entirely unrelated to the attenuation of phosphorylation of Cbl and Syk. SHP-2 also functions as an adaptor protein, and tyrosine phosphorylation of SHP-2 was shown to be important for maximal ERK phosphorylation induced by some growth factors [223, 224]. Our observation in Chapter 3 that LPS-induced ERK phosphorylation is strictly dependent on TLR4 (Figure 3.6), and the fact that LPS-induced activation of ERK is uncoupled from p21Ras activation [177], fits with the observations of Araki et al. that association of SHP-2 and Grb2 (an upstream adaptor in the Ras pathway) is insufficient to promote full ERK activation in response to growth factors [223]. Although SHP-2 has been implicated upstream of Ras in several pathways [224, 225], a role for SHP-2 in LPS-induced activation of p21Ras can be ruled out, as SHP-2 phosphorylation does not occur until after ten minutes of LPS treatment whereas p21Ras activation begins by one minute following LPS treatment in wild-type macrophages (Figure 3.4C).

SHP-2 was also shown to play a role in focal adhesions and cytoskeletal modifications. IL-1 signalling, which shares the MyD88 adaptor pathway with TLRs, stimulates SHP-2
phosphorylation, and blocking the activity of SHP-2 inhibits IL-1-induced actin filament assembly and cell contraction [226]. Loss of function studies revealed roles for SHP-2 in regulating actin filaments, focal adhesion complexes, cell spreading and cell motility [227-229]. Tyrosine phosphorylation of paxillin, a focal adhesion-associated scaffolding protein, plays a role in various cytoskeletal pathways, including cell adhesion [230], cell spreading [231, 232] and cell motility [233]. Thus, LPS-induced, TLR4-dependent phosphorylation of SHP-2 and paxillin could contribute to the differences we observed between wild-type and TLR4-deficient cells during migration in vitro and during leukocyte recruitment in vivo.

The morphological changes induced by LPS in TLR4-deficient macrophages confirmed a functional role for LPS-induced tyrosine phosphorylation. However, it must be noted that LPS triggered these morphological changes in macrophages lacking TLR4 only when higher concentrations of LPS (5 μg/mL) were used, whereas wild-type and CD14-deficient macrophages displayed cell spreading and actin cytoskeleton rearrangements at much lower doses of LPS. The reliance on higher concentrations of LPS to achieve effects in cells lacking TLR4 could be due to a number of factors. One possibility is that the receptor(s) involved in mediating LPS effects in absence of TLR4 could have a lower affinity for LPS than TLR4. Our observation that macrophages lacking both TLR4 and CD14 do not respond to LPS by inducing cell spreading and actin cytoskeleton rearrangements, in contrast to the responses of wild-type, TLR4- and CD14-deficient macrophages, demonstrate that either TLR4 or CD14 is sufficient to trigger these LPS-induced responses in vitro. In the case of TLR4-deficient macrophages, CD14 is capable of mediating LPS-induced morphological changes only when there are higher concentrations of LPS available. Consistent with the
idea that TLR4-independent signalling may be mediated by a receptor with lower affinity for LPS than TLR4, CD14 does indeed have a lower binding affinity for LPS than TLR4 [234]. This is not unexpected for a protein that functions to pass off captured molecules to nearby receptors.

Another possibility that could account for the necessity of higher LPS concentrations to induce responses in TLR4-deficient macrophages could be that, in absence of TLR4, there is the requirement for a critical concentration of LPS to ensure the formation of micelles, or aggregates, of LPS. These aggregates would then be capable of clustering receptors and may function similarly to the array of LPS found on the surface of intact bacteria. There is some controversy over whether LPS aggregates or monomers are better able to activate cells. Takayama et al. reported that dis-aggregated LPS was more efficient in stimulating the B cell line 70Z/3 than the aggregated form [235]. There are other reports, however, that demonstrate the importance of LPS aggregates as biologically active units. Mueller et al. showed that human mononuclear cells presented with the same concentration of lipid A, either as monomer or as aggregates, strongly induced TNF production in response to lipid A aggregates, but not monomers [236]. More recently, Sasaki et al. demonstrated that LPS monomers and aggregates are both biologically relevant, hypothesizing that they stimulate different pathways [237]. Thus, it is not unlikely that, depending on the cell type and pathway under investigation, LPS monomers or aggregates may induce very different effects. In addition, there are reports demonstrating that the presence of lipoproteins in serum shift the critical multimerization concentration of LPS, necessitating higher concentrations of LPS to initiate aggregation [237]. Our experiments, performed in the
presence of serum, may require higher concentrations of LPS to overcome the solubilization of LPS by serum proteins such as LBP and soluble CD14.

In line with the notion that arrayed particles have functionally different effects than soluble particles, Goodridge et al. demonstrated that Dectin-1 signalling required its ligand beta-glucan to be arrayed as a particulate, similar to that found on the surface of a phagocytosable particle such as a yeast cell [238]. This group found that beta-glucan, either immobilized on tissue culture plates or on polysterene latex beads, initiated Dectin-1 signalling, whereas soluble beta-glucan did not. It is tempting to speculate that initiation of signalling via CD14 requires repeating units, such as in aggregates or on the surface of bacteria, to cluster membrane-bound CD14, and that this clustering disturbs the lipid rafts in such a way that local SFKs get activated. Indeed, there are studies indicating that ligation of CD14 or other GPI-linked proteins is sufficient to induce SFKs [107, 108]. However, the possibility that CD14 is critically important as a co-receptor for another LPS signalling molecule, in addition to TLR4, and not as a signalling receptor on its own, cannot be ruled out.

Our identification of TLR4-independent pathways prompts consideration of TLR-independent pathways for other TLR ligands, and indeed, there is some evidence for other TLR-independent pathways. Most serendipitous was the study by Sanjuan et al. that demonstrated TLR9-independent activity of CpG [239]. This group noted many of the same elements described here in this chapter: TLR9-independent, CpG induced tyrosine phosphorylation of Pyk2, Cbl, Vav and Syk, and subsequent cell spreading and actin
cytoskeleton rearrangements. Although they did not identify the receptor responsible for the TLR9-independent CpG responses, it is possible that CD14 may play a role. CD14 has since been identified as an important co-receptor for TLR9; CD14 associates with CpG and is required for efficient phagocytic uptake of CpG in macrophages [240]. Taken together with our data demonstrating that CD14 is required for maximal tyrosine phosphorylation induced by LPS and that CD14 can play a role in actin cytoskeleton rearrangements in absence of TLR4, CD14 remains a good candidate for participating in TLR9-independent, CpG responses as well as in TLR4-independent, LPS responses.

The TLR4-independent effects we observed in mice injected with LPS are of particular significance, as they demonstrate the functional importance of the TLR4-independent pathways in vivo. Using our ultra-pure LPS, we confirmed the early recruitment of neutrophils to the peritoneal cavities of TLR4- or CD14-deficient animals, but not wild-type animals, six hours after LPS injection. Consistent with this, Andonegui et al. demonstrated that neutrophils are recruited to the lungs of wild-type mice within four hours of intraperitoneal LPS injection [241]. Intriguingly, this group also showed that TLR4 on the endothelium, not the leukocytes, was critical for this response. They found that TLR4-deficient neutrophils still trafficked to the lungs following LPS injection, provided that the endothelial cells expressed TLR4. Taken together with our results, this would suggest that in CD14-deficient animals, some trafficking of neutrophils to the lung may occur. However, in mice lacking both TLR4 and CD14, neutrophil recruitment to the peritoneum is diminished, indicating a role for CD14 in this response as well. Thus, CD14 may play a role both in the TLR4-dependent recruitment of neutrophils away from the peritoneal cavity and
in the TLR4-independent recruitment of neutrophils to the peritoneal cavity following LPS treatment. In contrast, CD14 does not play a role in the recruitment of monocytes to the peritoneal cavity at this time point. However, in both cases, the LPS responses observed in mice deficient in both TLR4 and CD14 indicate there are additional LPS-binding proteins acting to recruit neutrophils and monocytes to the peritoneal cavity.

The response of peritoneal macrophages to LPS is particularly noteworthy, as LPS induced similar changes in both wild-type and TLR4-deficient animals. Although the extent of macrophage loss is not quite that observed in wild-type animals, there was still a significant decrease of macrophages in the peritoneal fluid from TLR4-deficient animals. This indicates that although TLR4 can contribute to this response, the loss of macrophages from the peritoneal fluid is predominantly TLR4-independent, validating the relevance of the TLR4-independent pathway in vivo. Although we have not formally investigated the loss of macrophages from the peritoneal cavity six hours after LPS administration, it is tempting to speculate that the macrophages have not migrated out of the peritoneal cavity, but instead have adhered tightly to the peritoneal wall. We observed LPS-induced cell spreading and cytoskeletal rearrangements in vitro, suggestive of cell adhesion [242], and LPS stimulates adhesion in multiple cell types [243]. Indeed, this is consistent with our observation that BM-derived macrophages from wild-type and TLR4-deficient mice, treated with LPS to induce cell spreading and actin cytoskeleton rearrangements, are much more difficult to remove from the tissue-culture treated plastic than untreated macrophages generated from either wild-type or TLR4-deficient mice. In vitro, we observed complete loss of LPS-induced cell spreading and cytoskeletal rearrangements in macrophages lacking in both TLR4 and
CD14. *In vivo*, however, animals deficient in both TLR4 and CD14 still responded to LPS with a loss of macrophages from the peritoneal fluid. That macrophage responses *in vivo* do not mimic macrophage responses *in vitro* is not surprising, as there are many resident cell types that may respond to LPS and mediate the responses of resident macrophages by secondary effects, perhaps by producing cytokines or chemokines. Thus, in the case of macrophage responses *in vivo*, similar to that of neutrophil and monocyte responses, there is still evidence for at least one other signalling receptor mediating responses to LPS.

In summary, we show here further evidence that LPS can signal independently of TLR4 and that the TLR4-dependent pathway negatively regulates some TLR4-independent signals. The phosphorylation of SFK targets with roles in cytoskeleton rearrangements, particularly Cbl, CrkL, Pyk2, Vav and Syk, did not require TLR4. Consistent with a role for the TLR4-independent pathway in cytoskeletal dynamics, in the absence of TLR4 we observed LPS-induced morphological changes in macrophages in vitro and recruitment of leukocytes in vivo. LPS did not trigger morphological changes in mice deficient in both TLR4 and CD14, providing additional support for our hypothesis that CD14 plays an important role in the TLR4-independent pathway in addition to its role in the TLR4-dependent pathway. However, LPS still triggered leukocyte recruitment in mice lacking both TLR4 and CD14, indicating that there are additional signalling receptors that contribute to LPS responses in vivo.
Chapter 5. Toll-like receptor ligands modulate cytokine receptors and signalling

5.1 Introduction

Macrophages are phagocytic cells that have critical roles in development, tissue homeostasis, innate immune responses, wound healing and inflammation (reviewed in [9]). Two key growth factors that regulate macrophage differentiation and function are colony-stimulating factor-1 (CSF-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF).

CSF-1 signals through an integral membrane tyrosine kinase, the CSF-1 receptor (CSF-1R, cfms), which it shares with IL-34 [244, 245]. Binding of CSF-1 to CSF-1R induces receptor dimerization and autophosphorylation of cytoplasmic tyrosine residues, leading to recruitment of various Src homology 2 (SH2)-domain containing proteins and subsequent activation of multiple downstream pathways [246-248]. Included among these are activation of p21Ras, PI3K, MAPK, phospholipase C (PLC) and various transcription factors (reviewed in [249]).

GM-CSF signals through the common β chain (βc), a subunit that it shares with IL-3 and IL-5 (reviewed in [250]). Binding of GM-CSF to GM-CSF-specific alpha chains triggers heterodimerization with βc chains. This brings βc-associated Janus kinases (JAKs) into close proximity, triggering activation of JAKs and subsequent phosphorylation of multiple tyrosine residues on the cytoplasmic domain of βc. These phosphotyrosine residues serve as docking sites for a variety of signal transducing proteins, including signal transducers
and activators of transcription (STATs), which are themselves phosphorylated by JAKs and/or SFKs. Other proteins are recruited to phosphotyrosine residues on βc via SH2-domains. Like CSF-1R signalling, signals downstream of βc lead to activation of p21Ras, PI3K, MAPK, PLC and multiple transcription factors (reviewed in [250, 251]). Both CSF-1 and GM-CSF regulate various aspects of macrophage function and behaviour, including survival, proliferation, differentiation and activation (reviewed in [252] and [253], respectively).

In Chapter 3 of this thesis, we showed that LPS-induced p21Ras activation was rapidly attenuated in wild-type macrophages, yet was sustained in TLR4-deficient macrophages. From these experiments, we concluded that signals downstream of TLR4 lead to the inhibition of p21Ras activation. This led us to hypothesize that TLR4 signalling might prevent the activation of p21Ras induced by stimuli other than LPS, either by inhibiting p21Ras itself or by interfering with elements upstream of p21Ras activation. As CSF-1 and GM-CSF signal through different growth factor receptors to activate p21Ras, we investigated whether LPS treatment could prevent the activation of p21Ras by these growth factors.

We show in this chapter that pretreatment of wild-type, bone marrow-derived macrophages with LPS indeed prevented CSF-1 and GM-CSF from activating p21Ras, but did not interfere with phorbol 12-myristate 13-acetate (PMA)-mediated activation of p21Ras. Investigating mechanisms upstream of p21Ras, we found that LPS and other TLR ligands induced cleavage of CSF-1 receptor through the activity of metalloproteases. TLR signalling also induced a mobility shift of βc, although unlike activation of p21Ras, other signalling...
events induced by GM-CSF, including tyrosine phosphorylation of βc, STAT5 or SHP-2, were not altered by pretreatment with TLR ligands. Inhibition of the activities of both MAPK kinases 1 and 2 (MEK1/2) and p38 MAPK prevented CSF-1R cleavage induced by LPS, but did not completely prevent the mobility shift of βc. However, the inhibition of MEK1/2 and p38 MAPK together was sufficient to restore p21Ras activation induced by either CSF-1 or GM-CSF after LPS treatment. Thus, TLR signalling blocks CSF-1 and GM-CSF-mediated p21Ras activation through MAPK-dependent pathways.

5.2 Results

5.2.1 TLR4 signalling prevents p21Ras activation by IL-3, GM-CSF and CSF-1

To test whether TLR4 signalling affects the activation of p21Ras by other stimuli, we pretreated BM-derived macrophages with LPS prior to stimulation with a variety of ligands known to induce p21Ras activation. We found that pretreatment with LPS prevented p21Ras activation induced by IL-3, GM-CSF and CSF-1, but did not block p21Ras activation induced by PMA (Figure 5.1A). Pretreatment of TLR4−/− macrophages with LPS did not block IL-3- or CSF-1-induced p21Ras activation (Figure 5.1B), confirming the requirement of TLR4 for this inhibitory pathway. From these experiments, we concluded that TLR4 signalling does not inhibit p21Ras itself, and that the block in p21Ras activation occurs upstream of p21Ras activation by the cytokines CSF-1, GM-CSF and IL-3.
5.2.2 TLR4 signalling induces CSF-1R cleavage

Studies by others have described LPS-induced downregulation of CSF-1R in several macrophage models, including peritoneal macrophages [254] and the macrophage-like cell lines BAC-1.2F5 [255] and p388D1 [256]. Accordingly, we examined whether LPS triggered downregulation of CSF-1R in our BM-derived macrophages. We found that indeed, LPS treatment of wild-type macrophages lead to the disappearance of the mature CSF-1R (Figure 5.2A) and that the TLR adaptor MyD88 and the TLR4 co-receptor CD14 both contribute to this disappearance (Figure 5.2B & C). MyD88 and CD14 are not strictly
required, however, as at later time points LPS induces the disappearance of CSF-1R in both MyD88- and CD14-deficient macrophages. Although LPS treatment triggered the loss of mature CSF-1R, levels of CSF-1R precursor were unaffected by LPS treatment (Figure 5.2A, B & C). This suggested that the disappearance of CSF-1R was due to degradation or cleavage rather than down-regulation of its synthesis. Consistent with this, treatment of wild-type macrophages with the metalloprotease inhibitor GM6001 prevented the LPS-induced disappearance of CSF-1R and limited the accumulation of the ~55 kDa intracellular cleavage fragment (ICF) (Figure 5.2D). Together, these experiments provide evidence that LPS, signalling via TLR4, induces cleavage of CSF-1R in BM-derived macrophages through the activity of metalloproteases.
Figure 5.2. TLR4 signalling induces CSF-1R cleavage.

BM-derived macrophages from C57BL/6 wild-type and TLR4<sup>−/−</sup> (A), MyD88<sup>−/−</sup> (B) or CD14<sup>−/−</sup> (C) mice were stimulated with 5 μg/mL K12 LPS for indicated times. (D) BM-derived macrophages from C57BL/6 wild-type mice were treated with 10 μM GM6001 for 1 hour prior to stimulation with 5 μg/mL K12 LPS for indicated times. Lysates in all panels were immunoblotted for CSF-1R. Equal loading was confirmed (not shown).
5.2.3 TLR4 signalling induces modification of βc

The cleavage of CSF-1R triggered by LPS could account for the block in CSF-1-induced p21Ras activation we observed in macrophages pretreated with LPS. We also found that pretreatment with LPS blocked p21Ras activation induced by GM-CSF and IL-3 (Figure 5.1A). GM-CSF and IL-3 share a common signalling receptor subunit, βc, with IL-5. Interested in whether LPS might be affecting GM-CSF and IL-3 signalling at the level of the receptor, analogous to our observations with CSF-1R, we evaluated βc expression in macrophages following LPS stimulation. Although the protein levels of βc did not appear to change after LPS treatment, LPS did induce a shift in the electrophoretic mobility of the βc, retarding its migration through the SDS-PAGE gel (Figure 5.3A). Similar to our observations with LPS-induced CSF-1R degradation, we found that MyD88 and CD14 both contribute to the mobility shift of the βc at early time points but are dispensable at later time points (Figure 5.3B & C). The mechanism of the modification of βc by LPS will be explored in section 5.2.5.
Figure 5.3. TLR4 signalling induces an electrophoretic mobility shift in βc.
BM-derived macrophages from C57BL/6 wild-type and TLR4+/− (A), MyD88+/− (B) or CD14+/− (C) mice were stimulated with 5 μg/mL K12 LPS for indicated times. Lysates in all panels were immunoblotted for βc. Equal loading was confirmed (not shown).

5.2.4 TLR signalling induced by other TLR ligands triggers CSF-1R cleavage and modification of βc

We next examined whether pretreatment with agonists for TLRs other than TLR4 could also block p21Ras activation by CSF-1 and GM-CSF or IL-3. We found that pretreatment with ligands for TLR2 (lipoteichotic acid; LTA), TLR9 (CpG oligonucleotides) and TLR3 (poly(I:C)) all prevented activation of p21Ras by CSF-1 (Figure 5.4A). Poly(I:C) treatment also prevented activation of p21Ras induced by IL-3 and GM-CSF (LTA and CpG were not tested). Similar to LPS, treatment of macrophages with LTA, CpG or poly(I:C) triggered CSF-1R cleavage (Figure 5.4B) and induced an electrophoretic mobility shift of the βc
(Figure 5.4C), confirming that multiple TLR ligands modulate cytokine receptors and signalling.

Figure 5.4. Other TLR ligands block p21Ras activation by cytokines and modulate cytokine receptors.

BM-derived macrophages from C57BL/6 wild-type mice were treated with LTA (1 μg/mL), CpG ODN 1668 (1 μM), poly(I:C) (25 μg/mL) or K12 LPS (100 ng/mL) for 30 minutes, or left untreated. (A) Where indicated, macrophages were stimulated with 10 ng/mL of specified cytokine (CSF-1, IL-3 or GM-CSF) for 5 minutes, either directly or after stimulation with TLR ligand. Lysates were subjected to pull-down assay for activated Ras and immunoblotted for p21Ras. (B) Lysates were immunoblotted for CSF-1R. (C) Lysates immunoblotted for βc. Equal loading was confirmed (not shown).
5.2.5 Modification of βc by TLR ligands

The electrophoretic mobility shift we observed for βc following TLR signalling implied that βc was being modified in such a way as to impede its migration through the SDS-PAGE gel. Modifications that could affect mobility include changes in charge or attachment of small species to the receptor. Changes in phosphorylation state can greatly affect the charge of protein, so we examined whether LPS triggered phosphorylation changes on βc. Using immunoprecipitation of the βc and phosphoserine and phosphotyrosine antibodies, we ruled out tyrosine and serine phosphorylation as the cause of the βc mobility shift (Figure 5.5). Covalent attachment of small proteins such as ubiquitin proteins or small ubiquitin-like modifier (SUMO) proteins could also alter the molecular weight of a protein, slowing its migration through an SDS-PAGE gel. IL-5 signalling was reported to induce ubiquitination of βc [257, 258] and TLR signalling activates ubiquitin ligases that are critical components of TLR signalling pathways [259, 260]. TLR7 signalling has been reported to induce SUMO modification of components of the TLR signalling machinery [261]. However, preliminary experiments using immunoprecipitation of βc and antibodies for ubiquitin suggest that ubiquitination does not contribute to the βc mobility shift (data not shown). Further experiments using the SUMO inhibitor anacardic acid indicate that SUMOylation is also not the cause of the βc mobility shift (preliminary results, data not shown). We also attempted to use mass spectrometry to identify the modification of βc, however, we were only successful at generating a small fragment of βc via mass spectrometry, and this fragment did not contain any modified residue(s) (data not shown).
Figure 5.5. Phosphorylation on serine or tyrosine residues on βc are not responsible for βc mobility shift.
BM-derived macrophages from C57BL/6 mice were treated with 100 ng/mL K12 LPS for 30 minutes or with 10 ng/mL GM-CSF for 5 minutes. Lysates were immunoprecipitated with anti-βc antibodies and immunoblotted with the anti-phosphoserine antibody 4A4 and then with the anti-phosphotyrosine antibody 4G10. The immunoblot was stripped and re-probed with anti-βc antibody to confirm equal immunoprecipitation.

5.2.6 TLR-induced modification of βc does not impair the initiation of βc signal transduction

Unsuccessful at identifying the modification on βc, we next examined whether any other pathways downstream of βc, other than the Ras pathway, were affected by TLR signalling. We first examined whether the modification of βc affected the ability of GM-CSF to bind the GM-CSF receptor complex and initiate signal transduction. Macrophages pretreated with LPS were stimulated with GM-CSF and βc was immunoprecipitated from lysates and examined for tyrosine phosphorylation. Pretreatment with LPS did not impair the tyrosine phosphorylation of βc, indicating that the modified βc was still at the cell surface and could heterodimerize with ligand-bound alpha chains to activate JAKs (Figure 5.6A). STAT5 phosphorylation induced by GM-CSF was also not impaired by pretreatment with LPS, confirming that modified βc still efficiently activate JAKs (Figure 5.6B).
2 to βc leads to tyrosine phosphorylation of SHP-2 and promotes its association with the adaptor protein growth factor receptor-bound protein 2 (Grb2) and the Ras GEF SOS [262, 263]. To see if this pathway was impaired, we next examined SHP-2 phosphorylation following GM-CSF stimulation in macrophages pretreated with LPS. Using Grb2 as bait in a pull-down assay, we precipitated phosphorylated SHP-2 after GM-CSF stimulation, even when cells were pretreated with LPS (Figure 5.6C, upper panel). Interestingly, we also observed a shift in the electrophoretic mobility of SOS following LPS treatment, suggesting that SOS also undergoes LPS-induced modification (Figure 5.6C, lower panel). From these experiments, it was clear that the LPS treatment does not impair all GM-CSF-induced signals.
Figure 5.6. Mobility shift of βc does not affect initiation of βc signal transduction.
BM-derived macrophages from C57BL/6 wild-type mice were treated with 100 ng/mL K12 LPS for indicated times or left untreated. Where indicated, macrophages were stimulated with 10 ng/mL of GM-CSF for 5 minutes, either directly or after 30 minutes of LPS treatment. (A) βc was immunoprecipitated from lysates using anti-βc antibodies and immunoblotted with anti-phosphotyrosine antibody 4G10. The immunoblot was stripped and re-probed with βc to confirm equal immunoprecipitation. (B) Lysates were immunoblotted for phospho-STAT5 (Tyr694). The immunoblot was stripped and reprobed with actin to confirm equal loading. (C) Lysates were subjected to pull-down assay using GST-Grb2 as bait and blotted for both phospho-SHP-2 (Tyr542) (upper panel) and SOS1/2 (lower panel).

5.2.7 TLR-induced CSF-1R cleavage, βc mobility shift and inhibition of p21Ras activation require activation of MAP kinases

We next examined the molecular mechanism by which LPS modulates CSF-1R and βc. Studies using p388D1 cells showed that inhibiting MEK activity limited, but did not completely prevent, CSF-1R cleavage induced by LPS [264]. Another group found that inflammatory stimuli such as IL-1 and LPS blocked IL-6-induced STAT3 phosphorylation in human macrophages, and that p38 MAPK activity was required for the IL-1-mediated effect on IL-6 signalling [265]. Accordingly, we used inhibitors of MEK1/2 and p38 MAPK activities, U0126 and SB203580 respectively, to evaluate the contribution of these MAPKs to LPS-induced CSF-1R cleavage and βc modification. In contrast to Glenn et al. [264], we found that inhibiting MEK1/2 alone did not affect LPS-induced CSF-1R cleavage; nor did it affect
LPS-induced modification of βc. Inhibiting p38 MAPK did not affect βc modification, but did somewhat limit CSF-1R cleavage. Treating macrophages with both inhibitors, however, completely inhibited LPS-induced CSF-1R cleavage and limited the mobility shift of βc. Although the more quickly migrating bands of βc still disappeared with LPS treatment, the more slowly migrating species did not accumulate when cells were cultured with both U0126 and SB203580 (Figure 5.7A, upper panels). p38 MAPK phosphorylation induced by LPS was not affected by the presence of these inhibitors, indicating that the potent effect of these two inhibitors together was not due to general toxicity (Figure 5.7A, bottom panel). Thus, TLR signalling in BM-derived macrophages, through the activation of the MEK and p38 MAPK pathways, modulates CSF-1R and βc.

To determine whether inhibition of MEK1/2 and p38 MAPK was sufficient to restore CSF-1 and GM-CSF-induced p21Ras activation, we cultured BM-derived macrophages with a combination of U0126 and SB203580 prior to treatment with LPS, and then followed with cytokine stimulation. We found that, indeed, inhibition of both MEK1/2 and p38 MAPK was sufficient to restore CSF-1 and GM-CSF-induced p21Ras activation in macrophages after LPS treatment (Figure 5.7B). These results demonstrate that TLR signalling inhibits p21Ras activation induced by CSF-1 and GM-CSF through a pathway dependent on both Erk and p38 MAPK activities.
Figure 5.7. Inhibition of both MEK and p38 MAPK blocks LPS-induced CSF-1R cleavage and βc mobility shift and restores p21Ras activation induced by CSF-1 and GM-CSF.

BM-derived macrophages from C57BL/6 wild-type mice were mock treated with DMSO or treated with 10 μM U0126 (Erk inhibitor) or 25 μM SB203580 (p38 MAPK inhibitor) or both inhibitors for 1.5 hours prior to stimulation with 100 ng/mL K12 LPS. Lysates were immunoblotted with indicated antibodies. (B) Where indicated, macrophages were stimulated with 10 ng/mL of CSF-1 or GM-CSF for 5 minutes, either directly or after 30 minutes of LPS treatment. Lysates were subjected to pull-down assay for activated Ras and immunoblotted for p21Ras.
5.3 Discussion

The experiments detailed in this chapter demonstrate that TLR signalling, in addition to limiting the duration of p21Ras activation induced by LPS (explored in Chapter 3 of this thesis), also impedes p21Ras activation by the cytokines CSF-1, GM-CSF and IL-3. We found that pretreatment with LPS did not impair p21Ras activation by PMA, indicating that the LPS-induced block in p21Ras activation by cytokines was upstream of p21Ras and not due to increase in GAP activity or impairment of p21Ras itself. In the case of CSF-1 signalling, we confirmed that TLR ligands induce the activation of metalloproteases and subsequent cleavage of CSF-1R. In the case of GM-CSF and IL-3 signalling, we noted TLR ligand treatment induced an electrophoretic mobility shift in the GM-CSF and IL-3 signalling receptor, βc, indicating some manner of modification of βc. However, modification of βc did not impair GM-CSF-induced receptor phosphorylation or phosphorylation of downstream targets other than Ras. We showed that LPS-induced cleavage of CSF-1R and modification of βc was delayed in cells lacking the TLR4 co-receptor CD14 or the TLR adaptor MyD88, indicating that CD14 and MyD88 play roles in the LPS-induced modulation of these cytokine receptors but are not critically important. Highlighting the non-critical role of MyD88, stimulation of TLR3—which does not use MyD88 as an adaptor—with its ligand poly(I:C) also induced efficient CSF-1R cleavage, modification of βc and blocked p21Ras activation by cytokines.

Our observation that TLR ligands induce CSF-1R cleavage in BM-derived macrophages was similarly described in other macrophage models, including peritoneal macrophages [254] and the macrophage-like cell lines BAC-1.2F5 [255] and p388D1 [256]. We confirmed
that blocking the activity of metalloproteases using the inhibitor GM6001 was sufficient to block TLR-ligand induced CSF-1R cleavage in BM-derived macrophages. This supports observations by others demonstrating that the metalloprotease TNF-alpha converting enzyme (TACE) mediates cleavage of CSF-1R induced by PMA [255, 266] or LPS [264] in macrophage-like cell lines.

A variety of stimuli, including cytokines, phorbol esters and bacterial products, are known to affect CSF-1R expression. IL-2, IL-4 and PMA were reported to induce downregulation of CSF-1R through pathways dependent on PLC and/or protein kinase C (PKC) [266-268]. Baccarini et al. [269] showed in bone marrow-derived macrophages that CSF-1-induced CSF-1R downregulation was PKC-independent but that CSF-1R downregulation induced by a mixture of LPS and interferon gamma (IFNγ) required PKC. Work by Glenn et al. [264], on the other hand, indicated that CSF-1R cleavage induced by treatment with LPS alone was PKC-independent, but instead was partially blocked when p388D1 cells were treated with the MEK1/2 inhibitor U0126. In our BM-derived macrophages, however, we found that MEK1/2 inhibition did not affect CSF-1R cleavage, whereas treatment with the p38 MAPK inhibitor SB203580 did partially inhibit LPS-induced CSF-1R cleavage. Using the inhibitors for MEK1/2 and p38 MAPK together, we were able to fully block LPS-induced CSF-1R cleavage and completely restore CSF-1-induced p21Ras activation in cells treated with LPS. Thus, although numerous cytokines and activating stimuli can trigger CSF-1R downregulation in macrophages, the pathways by which they do so are distinct and specific to both the stimuli—or combination of stimuli—and the macrophage cell-type under investigation.
TLR ligands also affected the GM-CSF and IL-3 signalling receptor, βc, by inducing an electrophoretic mobility shift, indicating some manner of modification of βc. Although we were unable to identify the TLR ligand-induced modification on βc, we were able to rule out tyrosine and serine phosphorylation and preliminary experiments suggest that ubiquitination and SUMOylation may also be ruled out. The LPS-induced appearance of the electrophoretic mobility shift of βc coincided with the block in GM-CSF and IL-3-induced p21Ras activation in LPS treated cells. However, modification of βc did not impair GM-CSF-induced receptor phosphorylation or block recruitment and phosphorylation of Stat5 or SHP-2. It is possible that the lower levels of phosphorylated SHP-2 observed after LPS treatment (Figure 5.6C) may not be sufficient to recruit the Grb2-SOS complex. It is also possible that the modification of βc may impair the recruitment of other scaffolding proteins that we did not investigate, for example Shc, which can also contribute to the recruitment of Grb2-SOS to βc [270].

However, we also cannot exclude the possibility that the modification of βc is not actually responsible for the defect in GM-CSF and IL-3-induced p21Ras activation. Instead, the LPS-induced block in p21Ras activation could be due to a general inhibition in the pathway upstream of p21Ras used by these cytokines. Several lines of evidence support this hypothesis.

Firstly, the LPS-induced block in p21Ras activation was limited to CSF-1, GM-CSF and IL-3, all of which recruit SOS to their respective signalling receptor [270, 271]. PMA, which activates the GEF Ras-GRP [272, 273], still strongly activated p21Ras after LPS treatment.
Interestingly, we observed an electrophoretic mobility shift in the Ras GEF SOS following LPS treatment (Figure 5.6C) that correlated with p21Ras inhibition. Various stimuli, including insulin [274, 275], osmotic shock [276] and EGF [275, 277, 278], were reported to induce an electrophoretic mobility shift in SOS that was due to MAPK-dependent serine/threonine phosphorylation of SOS. Phosphorylation of SOS coincided with downregulation of Ras activation in these studies, suggesting a negative feedback mechanism for Ras activation. In some cases, phosphorylation of SOS was observed to lead to uncoupling of the Grb2-SOS complex [274-276]. In other cases, phosphorylation of SOS did not affect its binding to Grb2, but instead prevented Grb2-SOS from forming complexes with growth factor receptors and the scaffolding proteins that link Grb2-SOS to the activated receptors [278, 279]. Consistent with a role for MAPK in regulation of p21Ras through the phosphorylation of SOS, we found that MEK1/2 and p38 MAPK inhibition did not fully prevent modification of βc, but was still sufficient to restore p21Ras activation by GM-CSF.

In addition, preliminary experiments with IL-6 seem to suggest that LPS treatment blocks IL-6-induced activation of p21Ras and that the IL-6 receptor complex is unimpaired (data not shown). These preliminary results would appear to support the hypothesis of a general inhibition of p21Ras, perhaps via the modification of SOS. Like CSF-1 and GM-CSF, IL-6 is thought to activate p21Ras following recruitment of Grb2-SOS to the IL-6 signalling receptor gp130 [280, 281]. However, further experiments must be done to confirm our initial observations concerning IL-6, LPS and p21Ras.
Modulation of cytokine signalling through modification or downregulation of cytokine receptors can have many consequences during infection. By affecting the receptor itself, there is the potential for impacting multiple downstream pathways. This is particularly significant for CSF-1 signalling, where LPS and other TLR ligands induce cleavage of CSF-1R, thereby limiting all signals downstream of CSF-1R. Dampening CSF-1-mediated signals may allow the macrophage to focus on signals transmitted through the various pattern recognition receptors and mount an efficient immune response.

There is also evidence that CSF-1 signalling directly impacts gene expression. A study by Sester et al [282] found that CSF-1 signalling repressed a certain subset of LPS-regulated genes. The downregulation of CSF-1R by LPS, and thus the dampening of CSF-1 signalling, permits the macrophage to upregulate a subset of LPS-responsive genes that have important functions in bacterial responses. As well, cleavage of CSF-1R could itself play a role in signal transduction. CSF-1R is a result of two cleavage steps at the cell membrane. The first cleavage step is mediated by TACE, and results in the shedding of the extracellular portion of the receptor [255, 264, 266]. The second cleavage is mediated by gamma secretase, and results in the release of the cytoplasmic domain of the receptor and accumulation of this fragment in the cytosol and nucleus before degradation by the proteasome [256]. This process is known as regulated intramembrane proteolysis and is a critical component of signal transduction for some receptors, in particular the Notch signalling pathway (reviewed in [283, 284]). Thus, it has been proposed by others that regulated intramembrane proteolysis of CSF-1R may also act as alternative signalling mechanism during infection [256, 264, 266].
Our observations with βc, however, suggest that the LPS-induced block in p21Ras activation by the cytokines CSF-1, GM-CSF and IL-3 could instead be a general inhibition of p21Ras activation. In this scenario, LPS prevents subsequent or continued activation of p21Ras by inhibiting signals upstream of p21Ras. Cleavage of CSF-1R could indeed account for the block in p21Ras activation and other CSF-1-induced signals. However, it should be noted that we have not tested whether blocking LPS-induced CSF-1R cleavage is sufficient to restore p21Ras activation. Although we show here that MAPK activities are critical for both LPS-induced cleavage of CSF-1R and the block of cytokine-induced p21Ras activation, we cannot rule out that these two events are independent of one another.

Taken together with our observations in Chapter 3, it appears that TLR signalling regulates p21Ras by preventing both its continued activation by LPS and its further activation by cytokines. One significant consequence of limiting p21Ras activation during infection may be to limit inflammation and thereby prevent the damage that chronic inflammation can inflict on local tissues and organs (reviewed in [285]). The p21Ras family members participate in many macrophage functions that contribute to inflammation, including cell survival [137, 138], proliferation [130-132] and cytokine production [142-145]. Consistent with a role for p21Ras in cell survival, in Chapter 3 we observed increased viability in TLR4-deficient macrophages treated with LPS, as compared to wild-type macrophages, which rapidly attenuate p21Ras activation. We also observed cleavage of cIAP2, a marker of apoptosis, in wild-type macrophages treated with LPS overnight, indicating that LPS, via TLR4, directly and/or indirectly triggers apoptotic signals in macrophages.
One way that TLR-induced apoptosis could contribute to immune responses is by preventing the spread of some intracellular pathogens during prolonged infections. TLR-induced apoptosis could also limit the length of time that local tissues are exposed to the inflammatory mediators secreted by macrophages, preventing tissue damage and excessive activation of local tissue by inflammatory cytokines. Studies examining loss-of-function and gain-of-function in the Ras pathway have implicated roles for Ras in induction of proinflammatory cytokines and chemokines, including IL-6 [142], IL-8 [143, 144] and TNF [145]. The production of such proinflammatory mediators is critical for appropriate immune responses to pathogens. However, excessive production of these proinflammatory species can have devastating consequences, inducing sepsis and, in some cases, systemic shock and death (reviewed in [286] and [287]). Therefore, negative regulation of p21Ras signalling by TLRs could also act as a mechanism to limit rampant cytokine production during infection and prevent the development of sepsis and septic shock. Thus, as p21Ras contributes to various facets of the inflammatory response, modulation of p21Ras activity by TLR signalling could impact the magnitude and extent of multiple inflammatory pathways and may have profound effects on inflammation.
Chapter 6. Lipopolysaccharide induces the expression of innate immune genes in the absence of Toll-like receptor 4

6.1 Introduction

Macrophage encounters with LPS result in the activation of a multitude of signalling molecules, including SFK, PI3K, p21Ras and MAPK [177]. These proteins are involved in signalling cascades linked to a variety of outcomes, including the activation of various transcription factors such as NFκB [150, 288-291], AP-1 [292-294] and activating transcription factor 2 (ATF2) [295, 296], among others. Cooperation of activated transcription factors triggers the induction of numerous genes that contribute to specific effector functions during infection [297].

A core transcriptional program induced by LPS was identified that included genes involved in growth and differentiation, cytoskeleton dynamics, phagocytosis, lipid metabolism, antigen presentation, apoptosis, oxidative stress, cytokine signalling, cell signalling and transcriptional regulation [297]. A key function of this core transcriptional program during infection is the production of inflammatory modulators, such as cytokines, chemokines and other secondary mediators that initiate inflammation. These mediators act to induce vasodilation, increase vascular permeability, trigger chemotaxis of circulating leukocytes, alter the adhesive properties of cells and induce fever [298]. Together, the various effector functions triggered by LPS contribute to the resolution of infection.
We observed previously that LPS, in the absence of TLR4, induces the activation of signalling proteins including p21Ras, Akt and various SFK targets, and also promotes viability and morphological changes in macrophages. These observations prompted us to hypothesize the existence of an alternative signalling receptor(s) for LPS and investigate whether LPS also induces gene expression in macrophages lacking TLR4. Microarray analysis of TLR4-deficient macrophages revealed that LPS induced the upregulation of genes involved in innate immune responses. We confirmed TLR4-independent, LPS-induced gene expression using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and investigated the role of CD14 in these responses.

6.2 Results

6.2.1 Transcript analysis of TLR4-deficient macrophages

To investigate the role of TLR4-independent signalling in LPS-induced gene expression, we performed a cDNA microarray analysis to examine gene induction in wild-type and TLR4−/− macrophages six hours after treatment with LPS. The LPS concentration used was 5 μg/mL of our ultra-pure LPS preparation from K12 E. coli, which we found activated p21Ras, Akt and various SFK targets, and also promoted viability and morphological changes in TLR4−/− macrophages, but did not elicit TNF production (see Chapters 3 and 4). Untreated wild-type and TLR4−/− macrophages showed similar patterns of gene expression (Figure 6.1, bottom four rows). Six hours of LPS treatment induced a global change in gene expression in wild-type macrophages (Figure 6.1, top two rows). Some genes were upregulated following LPS treatment (depicted in red, left panels of top two rows), while others were downregulated (shown in blue, right panels of top two rows). TLR4−/− macrophages treated with LPS
showed patterns of gene expression more similar to untreated macrophages than to wild-type macrophages stimulated with LPS; however, there were also distinct regions that showed alterations of gene expression following LPS treatment. Some of these genes were upregulated or downregulated in a manner similar to wild-type macrophages treated with LPS, while others were upregulated in TLR4⁻/⁻ macrophages after LPS treatment but not wild-type macrophages treated with LPS.

![Figure 6.1. Patterns of gene expression induced by LPS in wild-type and TLR4-deficient macrophages.](image)

BM-derived macrophages from C57BL/6 wild-type or TLR4⁻/⁻ mice were cultured for six hours in the presence of 5 μg/mL ultra-pure LPS from K12 *E. coli*. RNA was extracted from cell lysates and subjected to Affymetrix GeneChip Mouse Gene 1.0 ST Array analysis (n = 2). Relative gene expression values are depicted in a heat map generated by hierarchical clustering after gene expression was standardized to zero. Median values (zero) are depicted in grey, while upregulated genes have positive values (red) and downregulated genes have negative values (blue). Rows correspond to individual samples (8) and columns correspond to genes that showed changes in expression in TLR4⁻/⁻ macrophages after LPS treatment (966).
For the purposes of this study, we were most interested in genes that were positively regulated by LPS in TLR4−/− macrophages. We wished to focus on genes highly expressed in TLR4-deficient macrophages following LPS treatment, and thus set a cutoff of a minimum of four-fold increase in gene expression over levels in untreated macrophages. Below the four-fold cutoff, the expression levels of LPS were considerably higher in wild-type macrophages than in TLR4-deficient macrophages and thus were of less interest for this analysis. Based on this cutoff, we generated a list of 27 genes that were upregulated in TLR4−/− macrophage following LPS, and the results of this analysis are displayed in Table 6.1.
Table 6.1. Genes upregulated by LPS in TLR4-deficient macrophages.

<table>
<thead>
<tr>
<th>Gene</th>
<th>NCBI RefSeq</th>
<th>Fold change</th>
<th>Protein name &amp; function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Il1a</td>
<td>NM_010554</td>
<td>143.14</td>
<td>IL-1α (proinflammatory cytokine [299])</td>
</tr>
<tr>
<td>Il1b</td>
<td>NM_008361</td>
<td>66.40</td>
<td>IL-1β (proinflammatory cytokine [299])</td>
</tr>
<tr>
<td>Cxcl3</td>
<td>NM_203320</td>
<td>11.75</td>
<td>Chemokine (C-X-C motif) ligand 3; Macrophage inflammatory protein 2 beta; Growth-related oncogene gamma (monocyte chemoattractant [300])</td>
</tr>
<tr>
<td>Irg1</td>
<td>NM_008392</td>
<td>27.49</td>
<td>Immunoresponsive gene 1 (cellular metabolism enzyme [301])</td>
</tr>
<tr>
<td>Saa3</td>
<td>NM_011315</td>
<td>41.48</td>
<td>Serum amyloid A3 (acute phase inflammatory mediator, endogenous ligand for TLR4 [302])</td>
</tr>
<tr>
<td>Ptgs2</td>
<td>NM_011198</td>
<td>24.50</td>
<td>Prostaglandin-endoperoxide synthase 2/Cyclooxygenase 2 (COX-2) (prostaglandin synthesis pathway enzyme [303])</td>
</tr>
<tr>
<td>Fpr1</td>
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<td>19.83</td>
<td>N-formyl peptide receptor/Met-Leu-Phe receptor (chemoattractant receptor [304])</td>
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<tr>
<td>Gbp5</td>
<td>NM_153564</td>
<td>78.78</td>
<td>Guanylate binding protein 5 (promotes assembly of inflammasomes [305])</td>
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<tr>
<td>Tarm1</td>
<td>NM_177363</td>
<td>13.29</td>
<td>T cell-interacting, activating receptor on myeloid cells 1 (function n.d.)</td>
</tr>
<tr>
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<td>Uridine phosphorylase 1 (pyrimidine nucleoside phosphorylase [311])</td>
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<td>Rsad2</td>
<td>NM_021384</td>
<td>11.04</td>
<td>Radical S-adenosyl methionine domain-containing 2/ Virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible (Viperin) (anti-viral protein, inhibits viral replication [312])</td>
</tr>
<tr>
<td>Gene</td>
<td>NCBI RefSeq</td>
<td>Fold change WT</td>
<td>Fold change TLR4&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>Tet2</td>
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</table>

Values represent log<sub>2</sub> fold change over expression in untreated macrophages. All mRNA were expressed at similar levels in untreated wild-type and TLR4<sup>−/−</sup> macrophages. N.d., not described.

The genes induced by LPS in TLR4<sup>−/−</sup> macrophages predominantly coded for proteins with functions in innate immunity and included cytokines (IL-1α, IL-1β), chemoattractants (CXCL3, CCL5, CCL22), chemoattractant receptors (FPR1, CCRL2), interferon-regulated proteins (GBP5, GBP2, GBP7, IFIT2, IFIT3) and other immune regulators (IRG1, SAA3, COX-2, complement component 3, etc.). While the majority of genes identified in this
shortlist were upregulated by LPS more strongly in wild-type macrophages than TLR4−/− macrophages, some were induced to a more comparable extent in both wild-type and TLR4−/− macrophages (Il1b, lrg1, Fpr1, Rassf4). A notable exception was expression of Cxcl3, which was induced more highly in TLR4−/− compared to wild-type macrophages.

6.2.2 Validation of target genes identified by microarray analysis

We next sought to confirm the results of our microarray analysis on the top candidates using qRT-PCR. We selected eight genes that showed a greater than 30-fold increase in gene expression in TLR4−/− macrophages following treatment with LPS (Il1a, Il1b, Cxcl3, lrg1, Saa3, Ptgs2, Fpr1 and Ccl5). As the functions of neither Gbp5 nor Tarm1 were described at the time this study was performed, we elected not to evaluate their expression by qRT-PCR. We included Rassf4 in our analysis, as this gene appeared to be upregulated equally in wild-type and TLR4−/− macrophages by LPS in the array. We also included the cytokine genes Tnfa (TNFα), Il6 (IL-6) and Csf2 (GM-CSF) in this analysis, as we had not observed secretion of cytokines by TLR4−/− macrophages treated with LPS (Figure 3.1A and data not shown) and expected these genes to not be induced by LPS in TLR4−/− macrophages.

We observed induction of expression of all nine genes identified in the microarray in both wild-type and TLR4−/− macrophages treated for six hours with 5 μg/mL LPS (Figure 6.2A-I). As predicted by the microarray, the majority of our gene candidates were upregulated more highly in wild-type macrophages than in TLR4−/− macrophages (Figure 6.2A, B & D-I), although Il1a, Il1b and Fpr1 were strongly upregulated in both wild-type and TLR4−/−
macrophages (Figure 6.2A, B & G, respectively). Also consistent with our microarray prediction, Cxcl3 was more strongly induced by LPS in TLR4−/− macrophages than in wild-type macrophages (Figure 6.2C), indicating that TLR4 inhibits Cxcl3 expression at this time point. Interestingly, Tnfa, Il6 and Csf2 expression was also induced in TLR4−/− macrophages (Figure 6.2J, K & L, respectively). Tnfa and Il6 were upregulated more highly in wild-type macrophages, whereas Csf2 expression was induced more highly in TLR4−/− macrophages.
Figure 6.2. Validation of TLR4-independent gene expression induced by LPS.

BM-derived macrophages from C57BL/6x10 wild-type or TLR4−/− mice were cultured for six hours in the presence of 5 μg/mL ultra-pure K12 LPS (n = 3-4). RNA was extracted from cell lysates and qRT-PCR was performed. Gene expression was calculated relative to expression of RPII and values were normalized to the level of expression in untreated, wild-type macrophages. Results are expressed as mean ± SD. Two tailed, unpaired t-tests were performed to assess statistical significance of treatment conditions vs. untreated control.
### 6.2.3 CD14 plays a role in TLR4-independent, LPS-induced gene expression

Having confirmed LPS-induced gene expression in TLR4\(^{-/-}\) macrophages, we also wished to examine the effect of LPS dose on gene expression in TLR4\(^{-/-}\) macrophages. In addition to testing wild-type and TLR4\(^{-/-}\) macrophages, we also tested CD14\(^{-/-}\) and TLR4\(^{-/-}/CD14\(^{-/-}\) macrophages. As we previously established roles for CD14 in some TLR4-independent responses, specifically p21Ras activation (see Chapter 3) and tyrosine phosphorylation of some SFK targets and morphological changes (see Chapter 4) induced by LPS, we hypothesized that CD14 might also play a role in TLR4-independent gene expression.

We did not observe a strong relationship between the dose of LPS and gene induction in wild-type macrophages after six hours of treatment (Figure 6.3), suggesting that the concentration of LPS was saturating at the lowest dose of LPS (0.2 \(\mu\)g/mL). In contrast, gene expression in TLR4\(^{-/-}\) macrophages was only within the scope of wild-type or CD14\(^{-/-}\) responses when cells were treated with 5 \(\mu\)g/mL LPS (Figure 6.3A-C \& G; Il1a, Il1b, Cxcl3 and Fpr1). Although lower doses of LPS did induce some expression of our candidate genes in TLR4\(^{-/-}\) macrophages, the magnitude of induction was not significant by way of two-way analysis of variance (ANOVA) tests when analyzed with responses in wild-type and CD14\(^{-/-}\) macrophages (Figure 6.3). Interestingly, several genes showed increased expression in CD14\(^{-/-}\) macrophages, as compared to wild-type macrophages (Figure 6.3A-C, F, J \& K; Il1a, Il1b, Cxcl3, Ptgs2, Tnfa and Il6), suggesting that CD14 plays a role in dampening LPS-induced expression of these genes at this time point. Other genes showed more similar responses between wild-type and CD14\(^{-/-}\) macrophages (Figure 6.3D, E, G, I \& L; Irg1, Saa3, Fpr1, Rassf4 and Csf2), indicating that CD14 does not influence the
induction of expression of these genes when TLR4 is present. Notably, we observed little to no induction of gene expression for any of our target genes in macrophages lacking both TLR4 and CD14. This suggested that the gene induction observed in TLR4−/− macrophages after treatment with 5 μg/mL LPS was mediated by CD14, either signalling on its own or acting as a critical co-receptor for another LPS signalling receptor. For genes that were not strongly induced by LPS in TLR4−/− macrophages (Figure 6.3D-F & H-K; lrg1, Saa3, Ptgs2, Ccl5, Rassf4, Tnfa and Il6), however, TLR4 is clearly the dominant signal required for significant gene induction by LPS.
Figure 6.3. TLR4-independent gene expression induced after six hours of LPS treatment is dependent on LPS dose and the presence of CD14.

BM-derived macrophages from C57BL/6x10 wild-type, TLR4\(^{+/}\)CD14\(^{+/}\) or TLR4\(^{+/}\)/CD14\(^{+/}\) mice were cultured for six hours in the presence of indicated amounts of ultra-pure K12 LPS (n = 3-4). RNA was extracted from cell lysates and qRT-PCR was performed. Gene expression was calculated relative to expression of RPII and values were normalized to the level of expression in untreated, wild-type macrophages. Results are expressed as mean ± SD. Two-way ANOVA followed by Bonferroni post-hoc test was used to assess statistical significance of treatment conditions vs. untreated control.
6.2.4 TLR4 is required for sustained expression of the LPS-induced genes identified by microarray analysis

We also examined the expression of this same set of genes following 24 hours of LPS treatment. In wild-type macrophages, we observed gene expression induced by 24 hours of LPS treatment for all genes tested (Figure 6.4). Some genes were more highly expressed after 24 hours of LPS treatment (Figure 6.4A-C, E, G & L; Il1a, Il1b, Cxcl3, Saa3, Fpr1 and Csf2), while others were lower after 24 hours (Figure 6.4D, F & H-K; Irg1, Ptgs2, Ccl5, Rassf4, Tnfa and Il6), as compared to expression levels in wild-type macrophages after six hours of LPS treatment. In TLR4−/− macrophages, however, we observed little to no gene expression induced by LPS at the 24 hour time point. Although we observed a slight increase in Rassf4 (Figure 6.4I) and Tnfa (Figure 6.4J) expression after 24 hours treatment with the highest dose of LPS (5 µg/mL), these increases were not statistically significant. Thus, we concluded that TLR4 signalling is critical for sustained expression of the set of genes identified by our microarray analysis.
Figure 6.4. TLR4 is required for sustained gene expression following twenty-four hours of LPS treatment.
BM-derived macrophages from C57BL/6x10 wild-type, TLR4<sup>+/−</sup> CD14<sup>−/−</sup> or TLR4<sup>+/−</sup>/CD14<sup>−/−</sup> mice were cultured for 24 hours in the presence of indicated amounts of ultra-pure K12 LPS (n = 3-4). RNA was extracted from cell lysates and qRT-PCR was performed. Gene expression was calculated relative to expression of RPII and values were normalized to the level of expression in untreated, wild-type macrophages. Results are expressed as mean ± SD. Two-way ANOVA followed by Bonferroni post-hoc test was used to assess statistical significance of treatment conditions vs. untreated control.
6.2.5 Evidence against activation of NFκB in TLR4-deficient macrophages

In Chapter 3 of this thesis, we observed activation of p21Ras in both wild-type and TLR4\(^{-/-}\) macrophages following treatment with 5 \(\mu\)g/mL LPS, the same dose observed to induce TLR4-independent gene expression after six hours of LPS treatment (Figure 6.2). We found that the activation of p21Ras depended on the activities of SFK and PI3K. p21Ras \([150, 288]\), SFK \([290]\) and PI3K \([289]\) have all been implicated in the activation of NFκB, which can act as a transcription factor for the genes \(Il1a\) \([324]\), \(Il1b\) \([325]\), \(Ptgs2\) \([326]\), \(Ccl5\) \([327]\), \(Tnfa\) \([328]\), \(Il6\) \([329]\) and \(Csf2\) \([330]\). In its inactive state, NFκB is sequestered in the cytoplasm by NFκB inhibitor (IκB) proteins. Phosphorylation of IκBα leads to its degradation and release of NFκB, which then translocates to the nucleus where it can act as a transcription factor \([331]\). Using phosphorylation of IκBα as an indicator for NFκB activation, we examined wild-type and TLR4\(^{-/-}\) macrophages for IκBα phosphorylation at the same time points that we observed p21Ras activation. We observed phosphorylation of IκBα in wild-type macrophages following five minutes of LPS treatment and again after 30 minutes of LPS treatment (Figure 6.5). This biphasic phosphorylation of IκBα in wild-type cells is likely the result of resynthesis of IκBα and its subsequent rephosphorylation by the active TLR4 pathway. It’s possible that the first phosphorylation event at five minutes occurs downstream of the MyD88 pathway while the second phosphorylation at 30 minutes occurs downstream of the TRIF pathway following the resynthesis of IκBα. Regardless, we did not observe phosphorylation of IκBα at any of these time points in TLR4\(^{-/-}\) macrophages, suggesting that NFκB does not contribute to LPS-induced gene expression in TLR4\(^{-/-}\) macrophages.
Figure 6.5. TLR4 is required for phosphorylation of IκBα induced by LPS.
BM-derived macrophages from C57BL/6x10 wild-type and TLR4−/− mice were treated with 5 μg/mL ultra-pure K12 LPS for indicated times. Lysates were immunoblotted with phospho-IκBα (Ser32). Equal loading was confirmed (not shown).

6.2.6 LPS-induced expression of IL-1β pro-protein does not require TLR4

We next sought to confirm whether induction of gene expression correlated with induction of protein expression for those genes most highly expressed in TLR4−/− macrophages: Il1a, Il1b, Cxcl3 and Fpr1. Unfortunately, we were unable to obtain antibodies that recognized IL-1α, CXCL3 or FPR1, even in wild-type macrophages. However, we did observe expression of IL-1β pro-protein in both wild-type and TLR4−/− macrophages (Figure 6.6, top panel) following six hours of LPS treatment. IL-1β is first translated as an inactive pro-protein (31 kDa) that is cleaved to the active form (17 kDa) by activated caspase-1 [332]. Autoproteolytic cleavage of pro-caspase-1 (45 kDa) into activated caspase-1 (10 kDa) is initiated by the inflammasome [333]. We did not detect any of the active form of IL-1β in either wild-type or TLR4−/− macrophages (Figure 6.6 second panel from the top), suggesting that caspase-1 was not active in either macrophage population. Indeed, although we observed pro-caspase-1 in both wild-type and TLR4−/− macrophages (Figure 6.6, second panel from the bottom), we did not detect the active form of caspase-1 in either population of macrophages (Figure 6.6, bottom panel). From this we concluded that LPS treatment
alone was not sufficient to activate the inflammasome in either wild-type or TLR4−/− macrophages.

Figure 6.6. TLR4 is not required for IL-1β pro-protein production. BM-derived macrophages from C57BL/6x10 wild-type and TLR4−/− mice were treated with 5 μg/mL ultra-pure K12 LPS from for six hours. Lysates were immunoblotted with indicated antibodies. Equal loading was confirmed (not shown).

6.3 Discussion

The data presented here demonstrates that TLR4 is not strictly required for the induction of gene expression by LPS. Through our microarray analysis, we identified 27 genes in TLR4-deficient macrophages that were upregulated four-fold or greater in response to six hours of treatment with the same concentration of LPS that we found stimulated p21Ras activation in wild-type and TLR4-deficient macrophages (see Chapter 3). Selecting the eight genes most highly expressed by LPS-treated, TLR4-deficient macrophages in the microarray, we used qRT-PCR to confirm the upregulation of these genes in TLR4-deficient macrophages and found that CD14 influenced the expression of these genes. Finally, we also showed induction of IL-1β pro-protein expression but not active IL-1β in both wild-type and TLR4-
deficient macrophages, demonstrating that LPS can not only induce the expression of a subset of innate immune genes, but also induce protein expression independently of TLR4.

We observed that higher doses of LPS induced gene expression in the absence of TLR4. Although TLR4 is clearly important for inducing the maximal response of many of the genes identified by our analysis (Irg1, Saa3, Ptgs2, Fpr1, Ccl5 and Rassf4), other genes were induced as efficiently in wild-type and TLR4-deficient macrophages treated with 5 μg/mL LPS (Il1a and Il1b). We have hypothesized elsewhere that the relatively high concentration required to elicit TLR4-independent responses may be a consequence of signalling through a low affinity LPS receptor (or receptors) and/or the result of the requirement for the formation of aggregates of LPS that signal independently of TLR4. Such aggregates may more closely resemble the array of LPS on intact bacteria (see Chapter 4, Section 4.3 for further exploration of these hypotheses).

Our observation that Cxcl3 expression was induced in TLR4-deficient, but not wild-type, macrophages after six hour of LPS treatment indicates that TLR4 signalling acts to suppress or delay Cxcl3 expression, as we see induction of Cxcl3 expression by 24 hours of LPS treatment in wild-type macrophages. The negative regulation of TLR4-independent signals by TLR4 is a theme we observed multiple times during the studies presented in this thesis, both in vitro (e.g. TLR4 signalling limits the duration of LPS-induced activation of p21Ras and phosphorylation of certain SFK-target proteins) and in vivo (e.g. recruitment of neutrophils and monocytes to the peritoneal cavity six hours after LPS injection occurs only
in TLR4-deficient animals). Thus, we have abundant evidence that the TLR4-dependent pathway impacts the TLR-independent pathway.

Our qRT-PCR data for the six hours of LPS treatment revealed multiple roles for CD14. We found evidence for CD14 acting in its traditional role, that of a co-receptor for TLR4 that increases the efficiency of TLR4-induced responses under low concentrations of LPS [77]. Fpr1 and Ccl5 both appeared to rely on CD14 in this capacity, as CD14-deficient macrophages induced lower expression levels of these genes than wild-type macrophages at the lowest concentration of LPS.

Consistent with our previous observations of a role for CD14 in TLR4-independent activation of p21Ras, phosphorylation of SFK targets and morphological changes induced by LPS, we also identified a role for CD14 in mediating LPS-induced gene expression in the absence of TLR4. Lack of TLR4 and CD14 abrogated LPS-induced gene expression of all genes tested, indicating that CD14, signalling on its own or acting as a critical co-receptor for another LPS signalling receptor, participates in TLR4-independent gene induction. As well, the abrogation of responses in macrophages lacking both TLR4 and CD14 also lends further support to the purity of our LPS preparations.

Finally, we also observed a role for CD14 in dampening or delaying TLR4 signalling. Induction of Il1a, Il1b, Ptgs2, Tnfa and Il6 gene expression was greater in CD14-deficient macrophages than in wild-type macrophages. LPS also induced the expression of Cxcl3 in CD14-deficient macrophages, even at the lowest doses of LPS, whereas Cxcl3 expression
was not induced in wild-type macrophages at this time point. Together, these data suggest that CD14 negatively regulates the expression of this particular subset of genes following six hours of LPS treatment. This is in great contrast to the traditional, well-defined role of CD14 as a positive regulator of TLR4 signalling. Again, whether CD14 transduces signals itself or acts as a co-receptor for another LPS receptor to mediate these responses, remains to be evaluated.

LPS-induced gene expression in TLR4-deficient macrophages implies the activation of transcription factors via TLR4-independent pathways. Various transcription factors are activated downstream of p21Ras, SFK and PI3K, proteins which we observed to be activated by LPS in absence of TLR4. p21Ras effector pathways regulate activation of Elk1 [334], AP-1 [292], ATF2 [295], nuclear factor of activated T cells (NFAT) [292, 335] and NFκB [150, 288], among others. SFKs were also linked to NFκB activation [290] and were shown to activate STATs independently of JAKs [336] and to co-operate with PI3K and MAPK to activate AP-1 [293]. In addition, the PI3K/Akt axis was linked to NFκB activation [289] and to cyclic adenosine monophosphate response element-binding protein (CREB) activation [337]. However, it should be noted that the activities of MAPK also contributes to the activation of these transcription factors [291, 294, 295, 334, 335, 338] and we did not observe activation of MAPK in TLR4-deficient macrophages (Figure 3.6). Lack of MAPK activity also correlates with our observation of IκBα phosphorylation in wild-type macrophages only; however, we cannot rule out activation of IκBα outside of the time points we investigated.
Many transcription factors have been implicated in the regulation of each of the genes identified by our microarray, including those noted above that are activated downstream of p21Ras, SFK and PI3K. Induction of gene expression can be thought of as the result of the integration of multiple signalling pathways that contribute to the activation of various transcription factors that cooperate to activate downstream genes. We have identified distinct signalling pathways as either TLR4-dependent (MAPK) or TLR4-independent (p21Ras, SFK and PI3K), and transcription factor activation is implicated downstream of both pathways. Thus, it is conceivable that both TLR4-dependent and -independent signals are required for activation of the full complement of transcription factors and subsequent gene induction following LPS encounter. This hypothesis is supported by our data from mice lacking TLR4 and CD14, which showed little to no response to LPS. As well, the lack of MAPK activity in TLR4-deficient macrophages may contribute to the lower expression of many of our candidate genes in these macrophages, as MAPK have been implicated in the activation of many of the transcription factors noted above.

Although TLR4 was not required to initiate gene expression following six hours of LPS treatment, we found that it was required to maintain the expression of our candidate genes through 24 hours of LPS treatment. We detected TNF, IL-6 and GM-CSF in the supernatants of wild-type, but not TLR4-deficient macrophages treated with LPS (Figure 3.1A and preliminary results, data not shown), indicating that TLR4 is required for the secretion of these cytokines. Although sustained LPS signalling may play a role the expression of our candidate genes following 24 hours of LPS treatment, the persistence of gene expression in wild-type macrophages treated with LPS is likely to be at least partially
due to the autocrine effects of TNF, IL-6, GM-CSF and other cytokines secreted by wild-type macrophages. The decrease in LPS-induced gene expression in CD14-deficient macrophages, particularly for Ptgs2, Ccl5, Il6 and Csf2, also indicates a role for CD14 in the maintenance of expression of these genes. It is possible that CD14 plays an important role in LPS-induced signalling pathways that contribute to the induction of these genes at this time point. As we have identified roles for CD14 in both TLR4-dependent and -independent pathways, CD14 could be operating by either, or both, pathways to contribute to this effect. Another possibility is that lack of CD14 impairs the LPS-induced secretion of a subset of cytokines that contribute to the expression of these genes. Indeed, macrophages from CD14-deficient mice show impaired TNF and IL-6 release following LPS treatment [105].

The absence of MAPK activity in TLR4-deficient macrophages may also explain the lack of TNF, IL-6 or GM-CSF protein production in these macrophages. Work by others showed that TNF and IL-6 secretion by monocytes required ERK and p38 MAPK activities [339] and that inhibition of p38 MAPK blocked GM-CSF production [340]. The p38 MAPK pathway was also shown to play a role in mRNA stabilization of various cytokines, including TNF [341], IL-6, IL-1 and GM-CSF [342]. As well, the p38 MAPK pathway also plays a role in the post-transcriptional regulation of cytokines such as TNF and IFNγ following LPS stimulation [343]. Thus, it is not surprising that we were unable to detect TNF, IL-6 or GM-CSF in the supernatants of TLR4-deficient macrophages.

Roles for the p38 MAPK pathway in IL-1β transcription [344] and protein production [345] were also described; however, we found that LPS induced the production of IL-1β pro-
protein in TLR4-deficient macrophages, despite the lack of LPS-induced MAPK activity in these macrophages. Although we observed similar levels of IL-1β pro-protein in both wild-type and TLR4-deficient macrophages, we did not detect mature IL-1β in either population, as LPS failed to activate caspase-1 in both wild-type and TLR4-deficient macrophages. This observation is in agreement with studies that showed that LPS alone is insufficient to trigger activation of the inflammasome and subsequent activation of caspase-1 in macrophages [346].

Although we focused exclusively on genes that were upregulated by LPS in TLR4-deficient macrophages, there is additional data that may be obtained from this microarray data set. For example, we did not investigate any of the genes that were downregulated by TLR4-deficient macrophages following LPS stimulation. An analysis of those genes downregulated in both wild-type and TLR4-deficient macrophages and also those downregulated only in TLR4-deficient macrophages, could provide further insight into the consequences of TLR4-independent signalling and the interaction between the TLR4-dependent and TLR4-independent pathways.

In summary, the data shown here demonstrate that LPS, in absence of TLR4, can upregulate a subset of innate immune genes including cytokines, chemokines and other innate immune regulators, as well as induce IL-1β pro-protein production. Consistent with our previous observations, CD14 positively regulates the expression of both TLR4-dependent and TLR4-independent genes; however, we also observed a novel role for CD14 in dampening the expression of a subset of TLR4-dependent, LPS-induced genes. We
hypothesize that full induction of gene expression in wild-type macrophages requires the cooperation of both TLR4-dependent and -independent pathways.
Chapter 7. General discussion and future opportunities

The data in this thesis provide compelling evidence for the existence of alternative receptors that, in addition to TLR4, transduce signals from LPS in macrophages (Figure 7.1). We found that these TLR4-independent pathways are dependent on the activities of SFKs and PI3K and lead to the activation of p21Ras and various SFK targets including Cbl, CrkL, Pyk2, Vav and Syk. CD14 and members of class A and class B scavenger receptors were implicated in TLR4-independent signalling. We identified downstream pathways that are mediated by TLR4-independent pathways that include promotion of cellular viability, cell morphology and gene expression. Changes in leukocyte distribution in TLR4-deficient animals following LPS treatment confirmed that these TLR4-independent pathways also function in vivo. Furthermore, we identified a role for TLR4 in the negative regulation of TLR4-independent signalling, demonstrating that the TLR4-independent and TLR4-dependent pathways influence one another.
Figure 7.1. LPS signalling in macrophages: TLR4-independent and TLR4-dependent pathways.

LPS signals through CD14 and/or the scavenger receptors SR-AI/II and CD36 to activate SFK, PI3K and p21Ras. CD14 and the scavenger receptors may transduce signals on their own or may pass off LPS to another signalling receptor. Pathways downstream of TLR4-independent signalling include changes in cell morphology, enhanced viability and induction of genes such as II1α and II1β. Signalling via TLR4 leads to activation of MAPK family members and the NFκB pathway. TLR4 signalling also leads to inhibition of p21Ras through a MAPK dependent pathway. We hypothesize that the TLR4-mediated inhibition of Cbl phosphorylation also requires MAPK activity (light red dotted line).
The initial objective of this thesis was to resolve the controversy over whether LPS triggered activation of p21Ras in primary, bone marrow-derived macrophages. We found that LPS indeed induced the activation of p21Ras, but that this depended on the type (rough vs. smooth), dose and duration of LPS treatment. This observation provides insight into why the identification of p21Ras activation by LPS has proved so elusive: those studies that reported failure of LPS to stimulate activation of Ras [149] or to stimulate only very weak activation of Ras [189] used LPS from smooth strains of *E. coli*, which we found activated p21Ras much more weakly than LPS from rough strains of *E. coli*. We hypothesize that this effect may be due to the greater negative charge and greater hydrophobicity of rough LPS, as compared to smooth LPS. In addition, the rapid inhibition of LPS-induced p21Ras activation in wild-type macrophages may have also contributed to the difficulty in detecting its activation, particularly in studies looking at later time points.

Although many proteins are known to bind and modulate responses to LPS [80-104], interest in these proteins as LPS signalling receptors declined once TLR4 was identified as the gene responsible for impaired LPS responses in mice. Further experiments demonstrating the important role of TLR4 in cytokine production [72] also contributed to the reduced interest in other LPS binding proteins. However, as the work in this thesis shows, TLR4 is not the only receptor capable of transducing signals from LPS, and it is likely that many LPS binding proteins work together, each contributing their own set of signals that integrate to generate the global response to infection. This prompts questions as to the nature and purpose of TLR4-independent signalling. Chiefly, are TLR4-independent signals
a required component of the LPS signalling axis and, in absence of the signals generated by these alternative pathways, would signalling through TLR4 alone confer total responsiveness to LPS?

As a specific example, p21Ras activity was implicated in the induction of some proinflammatory cytokines and chemokines [142-145] and early experiments investigating the function of Ras in LPS responses suggested a positive role for p21Ras in LPS-induced cytokine responses [150, 152, 153]. We show in Chapter 3 that activation of p21Ras does not require TLR4; we observed similar levels of activated p21Ras in wild-type and TLR4-deficient macrophages following five minutes of LPS treatment. However, we also observed that wild-type macrophages showed greater activation of p21Ras than TLR4-deficient or CD14-deficient macrophages at very early time points following LPS stimulation, leading us to conclude that TLR4 may contribute to a more rapid activation of p21Ras. These observations raise questions about the ability of TLR4 alone to mediate LPS-induced activation of p21Ras. Although this question requires further investigation, two sets of preliminary experiments may provide some insight.

Firstly, prior to the initiation of the studies described in this thesis, we observed that the TLR4-agonist Taxol [347] failed to activate p21Ras in BM-derived macrophages. Secondly, we found no evidence for LPS-induced activation of p21Ras in BaF/3 cells stably transfected with murine TLR4/MD2 or TLR4/MD2/RP105. Although these results were extremely preliminary and require verification, they do make it tempting to speculate that TLR4 alone is not sufficient to activate p21Ras in response to LPS and that, by extension,
TLR4-independent pathways contribute absolutely critical components to LPS signalling. In addition, these same experiments, designed to test the ability of TLR4 alone to elicit p21Ras activity, could also be used to further investigate the impact of TLR4 signalling in isolation. For example, does TLR4 alone trigger phosphorylation of those SFK targets we found to be activated in absence of TLR4, and what are the responses of downstream pathways such as macrophage viability and morphological changes in the absence of other LPS-binding receptors?

During the writing of the thesis, two studies were published that described pathways that required delivery of LPS to the cytosol and led to activation of the inflammasome in the absence of TLR4 [348, 349]. These observations are distinct from ours, as we found no evidence for activation of the inflammasome in either wild-type or TLR4-deficient macrophages treated with LPS. However, this is not entirely surprising, as we did not artificially deliver LPS to the cytoplasm in our experiments. Interestingly, both groups also found that LPS treatment could trigger sepsis in TLR4-deficient animals that were pre-treated with TLR2 or TLR3 ligands. Although this phenotype was attributed to activation of caspase 11 and the inflammasome, it is worth noting that TLR2 or TLR3 stimulation would also trigger MAPK activity. In this thesis, we show that the MAPK pathway was not activated by LPS in absence of TLR4 and that this absence of MAPK activity correlated with lack of pro-inflammatory cytokine secretion. We hypothesize that addition of MAPK to our TLR-independent pathway could be sufficient to trigger the pro-inflammatory cytokine cascade that initiates sepsis and septic shock.
This thesis has revealed multiple functions for CD14 in macrophage responses to LPS. The current understanding is that CD14 operates as a co-receptor that passes off LPS to TLR4, increasing the sensitivity of TLR4 [350]. However, we found that CD14 mediates a subset of LPS responses, even in absence of TLR4. Although we did find evidence for CD14 operating in its traditional role of enhancing TLR4-dependent signalling, we also noted the involvement of CD14 in the negative regulation of some LPS-dependent signals. A study by Zanoni et al. [351] also supports a role for CD14 outside of TLR4 signalling in dendritic cells. They found that LPS-induced activation of NFAT in these cells was dependent on CD14 and not on TLR4, and that this pathway was important for regulating the dendritic cell life cycle. This is in contrast to our pathway in macrophages, where we observed that TLR4-independent signalling led to enhanced viability and that CD14 did not play a critical role in the TLR4-independent axis. However, Zanoni et al. did note distinct differences between macrophages and dendritic cells. Nonetheless, it is clear that the role of CD14 in LPS responses is much more complex than generally acknowledged.

In addition to CD14, we also observed roles for class A and class B scavenger receptors in our TLR4-independent, LPS signalling pathways. Like CD14, the scavenger receptors were also previously acknowledged as LPS binding proteins that contribute to LPS responses via TLR4 [352]. We showed in Chapter 3 that CD14 and scavenger receptors both contribute to LPS-induced p21Ras activation, which does not require TLR4. Mice lacking all of TLR4, CD14, SR-AI/II and CD36 would be extremely useful to identify whether other mechanisms, in addition to these receptors, also contribute to activation of p21Ras. Scavenger receptors also remain untested candidates for LPS-induced tyrosine phosphorylation of SFK and their
downstream targets. In addition, when we investigated leukocyte distribution following LPS injection *in vivo*, we found that mice lacking both TLR4 and CD14 still responded to LPS, indicating that there are additional receptors mediating leukocyte responses to LPS *in vivo*. Mice lacking TLR4, CD14, SR-AI/II and CD36 would be a necessary tool to investigate the contribution of scavenger receptors to *in vivo* responses, as we show that TLR4 signalling actually prevents early recruitment of neutrophils to the peritoneal cavity following injection of LPS.

The involvement of both CD14 and scavenger receptors in LPS signalling in absence of TLR4 is intriguing, as it is presently unclear how these receptors transmit signals. CD14 is a GPI-linked protein, without a transmembrane or signalling domain. Some studies reported the association of CD14 with SFK members [82]. In addition, ligation of multiple GPI-linked proteins was reported to activate SFKs co-localized on the inside of the plasma membrane [82, 107, 108]. Class A and class B scavenger receptors, on the other hand, do contain transmembrane domains; however, their short cytoplasmic tails lack identifiable signalling motifs and the mechanism by which they signal is not entirely clear [353]. Intriguingly, class B scavenger receptors were also reported to interact with SFK members [125, 126]. CD14 [354, 355], CD36 [356] and SR-A [357] were all identified in lipid rafts in macrophages, and it is possible that these receptors initiate signalling by clustering lipid rafts upon encounter with arrays of LPS. In line with this notion, CD14 and other lipid raft-resident receptors are hypothesized to recruit signalling molecules, including TLR4 and members of the TLR4 signalling complex, to lipid rafts following LPS stimulation [354, 355]. Nevertheless, we cannot rule out the possibility that CD14 and the scavenger receptors are acting as co-
receptors, passing off LPS to other cell surface receptors and/or facilitating the uptake and delivery of LPS to endocytic receptors.

Interestingly, both CD14 and scavengers are promiscuous receptors that bind a wide variety of polyanionic molecules, including various PAMPs. Thus, the TLR4-independent pathways we identified here may represent a pathway that is not actually specific for LPS, but rather a general mechanism for responding to various pathogenic stimuli. In line with this hypothesis, a study by Sanjuan et al. [239] reported SFK activity and cytoskeletal rearrangements induced by CpG through a TLR9-independent pathway. Their observations with CpG parallel several of our findings with LPS in Chapter 4, making CD14 an appealing candidate as an upstream receptor for their pathway as well. Furthermore, scavenger receptors are also attractive candidates as alternative CpG receptors, as both class A [358] and class B [359] scavenger receptors have been implicated in distinct CpG-induced signalling pathways, including cell adhesion and cytokine production.

The fundamental question is, of course, what is the purpose of these TLR-independent pathways for PAMP recognition, especially considering that the TLRs are much more sensitive receptors. The requirement for higher doses of LPS may indicate that these pathways could be important during conditions of increased pathogen load, perhaps acting as an extra enhancement in times of extreme danger. Likewise, TLR-independent signalling pathways would be of great advantage during conditions where TLRs have been downregulated [360] or the TLR-dependent pathway tolerized [361]. As highlighted earlier in this discussion, we hypothesize (1) that the TLR4-independent pathways may provide
critical signals that are necessary to induce a comprehensive response to LPS and (2) that these pathways may not be specific for LPS, but instead are a general mechanism for responding to various pathogenic stimuli. Thus, we propose a model in which TLR-independent pathways provide important, but broad signals to trigger response to infection, whereas the TLRs and other PRRs provide more specific signals that tailor the response to a particular class of pathogen.

In summary, the work in this thesis presents compelling evidence that refutes the current paradigm that LPS signals solely through TLR4. Both CD14 and class A and B scavenger receptors contribute to TLR4-independent signalling, which impacts cell morphology, cell survival and gene expression. We hypothesize that the proposed TLR4-independent pathways provide critical signals that are necessary for the overall response to LPS and that this pathway may represent a general mechanism for sensing various PAMPs. In addition, by contributing to the general knowledge of the pathways by which LPS signals, the work detailed here may aid the development of better strategies for treating sepsis and septic shock and subsequently save many human lives.
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