The Involvement of Interleukin-1 Receptor-Associated Kinase-1 (IRAK-1) as a Critical Modulator of Macrophage Migration

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ABSTRACT

Macrophage migration, an essential component of many biological processes and pathologic conditions, is mediated by integrated cellular signaling processes and cytoskeleton rearrangement. Recent advances indicate that the innate immunity signaling process plays a key role in the regulation of macrophage migration.

Furthermore, our lab has provided evidence demonstrating the involvement of a key innate immunity signaling kinase, IRAK-1, as a critical modulator of murine macrophage migration. Macrophage migration induced by a potent PKC activator, phorbol 12-myristate 13-acetate (PMA), or lipopolysaccharide (LPS) was significantly decreased in IRAK-1−/− murine macrophages compared with wild type cells. Mechanistically, we first demonstrated that IRAK-1 works downstream of PKCε and directly binds to VASP, a cytoskeleton regulatory protein, to regulate PMA-induced macrophage migration. Secondly, we proved that IRAK-1 is required for LPS-induced macrophage migration and expression of MCP-1, a chemotactic cytokine for macrophages, via transcription factor C/EBPδ instead of NFκB. IRAK-1 binds directly to IKKε and inhibition or knock-down of IKKε results in a significant decrease in C/EBPδ expression and MCP-1 mRNA expression. Lastly, we identified the direct association between IRAK-1 and a small signaling G protein, Rac1, a member of the Rac subfamily in the Rho family of GTPases. These findings further confirmed the essential role of IRAK-1 during macrophage migration. Our
research provides a novel facet regarding the molecular signaling processes regulating macrophage migration.
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LIST OF ABBREVIATIONS

BMDM: bone marrow-derived macrophage
CCR2: CC chemokine receptor 2
C/EBP: CCAAT/enhancer binding protein
Co-IP: co-immunoprecipitation
ChIP: chromatin immunoprecipitation
ECM: extracellular matrix
EST: expressed sequence tag
EAE: Experimental autoimmune encephalitis
Ena: *Drosophila*-Enabled
EVH1: Drosophila enabled vasodilator-stimulated phosphoprotein homology-1
GPCR: G-protein-coupled receptor
GAG: glycosaminoglycan
HBV: hepatitis B virus
IRF: interferon regulatory factor
IRAK-1: Interleukin-1 Receptor-Associated Kinase-1
IKK: IκB kinase
LPS: lipopolysaccharides
LRR: leucine-rich-repeat
MCP-1: monocyte chemotactic protein-1
MIF: macrophage migration inhibitory factor
MyD88: myeloid differentiation factor 88
OxLDL: oxidized low-density lipoprotein
PI3K: PI3-kinase
PKC: protein kinase C
PMA: phorbol 12-myristate 13-acetate
PAMP: pathogen-associated molecular pattern
Rac1: Ras-related C3 botulinum toxin substrate 1
SFK: Src-family tyrosine kinase
SH3: SRC homology3 domain
SNP: single nucleotide polymorphism
TIR: Toll/interleukin-1 receptor-like domain
TLR: Toll-like receptor
TCR: T cell receptor
TRAF6: tumor necrosis factor receptor-associated factor 6
TAK1: transforming growth factor β-activated kinase 1
VASP: vasodilator-stimulated phosphoprotein
WT: wild type
CHAPTER 1: INTRODUCTION

Anton van Leeuwenhoek, the pioneer of microscopy, first described the movements of bacteria in 1675, which then initiated studies on cell motility (Chicurel, 2002). No one would have been able to imagine that over 300 years later, the molecular understanding of this process would become a hot topic all over the world. Cell migration is a highly integrated process which plays an essential role in a variety of biological processes, including embryonic morphogenesis, immune responses, tissue repair, and homeostasis (Lauffenburger and Horwitz, 1996; Ridley et al., 2003). Aside from this, recent research has shown that cell migration also contributes to several important pathological processes such as tumor formation, atherosclerosis, and rheumatoid arthritis (Condeelis and Pollard, 2006; Gautier et al., 2009; Shahrara et al., 2009).

Growing evidence also suggests that cell migration has a close relationship with inflammation. The regulated expressions of chemokines, as well as chemokine receptors, during immune cell activation and migration have been extensively studied in the past (Baggiolini, 1998; Boldajipour et al., 2008). However, relatively little information has been available regarding the regulation of cell migration by signaling proteins in the innate immunity pathways. The migration of macrophages, an important component of innate immunity, plays a major role in the host response to inflammation, ischemia, infection, tumor progression, and tissue repair. Therefore, understanding the molecular mechanism of macrophage migration holds the promise of effective therapy.

IRAK-1, the most widely studied member of the IRAK family, is critically involved in the IL-1R/Toll-like receptor (TLR)-mediated signal transduction processes and therefore regulates
cellular innate immune responses and the resultant inflammation (Janssens and Beyaert, 2003). Additionally, there is evidence to suggest that IRAK-1 plays a critical role in systemic lupus erythematosus by mediating inflammatory cell infiltration into the glomeruli of the kidneys (Jacob et al., 2009). Moreover, IRAK-1-deficient mice exhibit a decreased monocyte/macrophage infiltration into inflamed brain tissues in the murine experimental autoimmune encephalitis (EAE) models compared to their wild type counterparts (Deng et al., 2003). These observations suggest a potential role for IRAK-1 in cellular functions and a potential link between immunity, cell motility, and inflammation.
CHAPTER 2: REVIEW OF LITERATURES

2.1 Regulations and Roles of the Interleukin-1 Receptor Associated Kinases (IRAKs) in Innate and Adaptive Immunity

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ABSTRACT

The interleukin-1 receptor associated kinases (IRAKs) are critically involved in the IL-1R/Toll-like receptor (TLR)–mediated signal transduction processes and therefore regulate cellular innate immune responses. Four IRAK members have been identified in the human genome (IRAK-1, 2, M, and 4), which seem to play distinct roles. Recent studies further suggest that some of the IRAK members may also participate in T cell and B cell signaling and regulate adaptive immunity. Given the critical and complex roles IRAK proteins play, it is not surprising that genetic variations in human IRAK genes have been found to be linked with various human inflammatory diseases. This review intends to summarize the recent advances regarding the biochemical regulations of various IRAK proteins and their cellular functions in mediating innate and adaptive immunity.
INTRODUCTION

Following the discovery of Toll-Like-Receptors (TLRs) and related downstream signaling molecules, the field of innate immunity and inflammation has recently drawn immense interest. Human hosts can specifically respond to distinct non-self molecules via TLRs and illicit complex yet specific responses including expression of cytokines and chemokines, cellular activation and proliferation (Li, 2004). Interleukin-1 receptor associated kinases (IRAK-1, 2, M, and 4) are intracellular kinases that can be differentially recruited to the TLR complex and mediate TLR signaling. Initially, it was thought that all IRAK proteins play similar and redundant function in activating transcription factor NFκB. However, several recent studies employing transgenic mice prove otherwise and indicate that each IRAK protein has distinct function (Huang et al., 2004; Kobayashi et al., 2002; Suzuki et al., 2002a). Biochemical analyses further reveal that distinct IRAK protein can be differentially modified and regulated (Kollewe et al., 2004; Su et al., 2006; Yamin and Miller, 1997). Furthermore, individual IRAK protein has distinct cellular and sub-cellular distribution pattern (Huang et al., 2004; Su et al., 2006). Intriguingly, selected IRAK molecules can also associate with protein partners involved in T cell and B cell receptor mediated signaling pathways, indicating that IRAK proteins are critical mediators for both innate and adaptive immune functions (Ohnuma et al., 2004; Suzuki et al., 2006). Studies employing transgenic mice as well as human population-based studies have revealed that genetic variations in various IRAK genes are linked with diverse diseases such as infection, atherosclerosis, sepsis, auto-immune diseases, and cancer (Arcaroli et al., 2006b; Cardenes et al., 2006; Deng et al., 2003; Huang et al., 2004; Yuasa et al., 2009) (Table 1).
IRAK-1

IRAK-1 was first identified by Cao et al. through biochemical purification of the IL-1 dependent kinase activity that co-immunoprecipitates with the IL-1 type 1 receptor (Cao et al., 1996). Micropeptide sequencing and subsequent cDNA library screening yielded a full length cDNA clone encoding a protein with 712 amino acids and a predicted molecular size of ~76KD. IRAK-1 message is expressed ubiquitously in diverse human tissues. By radiation hybrid analysis, Thomas et al. mapped the murine IRAK-1 gene to Xq29.52-q29.7 and human IRAK-1 gene to Xq28 (Thomas et al., 1999). IRAK-1 protein contains an N-terminal death domain, a central serine/threonine kinase domain, and a C-terminal serine/threonine rich region. There is also a putative nuclear location sequence (NLS at aa 503-508) and a nuclear exit sequence (NES at aa 518-526) (Su et al., 2006). Using human THP-1 cells, primary blood mononuclear cells, as well as mice splenocytes, we have demonstrated that there are two signature forms of IRAK-1; the unmodified 80KD form, and the modified 100KD form (Bear et al., 2000). IRAK-1 modification consists of phosphorylation, ubiquitination, and sumoylation (Kollewe et al., 2004; Su et al., 2006; Yamin and Miller, 1997). Depending upon the nature of its modification, IRAK-1 may perform distinct functions. The unmodified IRAK-1 may play a role in the activation of IRF3/7 as well as NFκB (Uematsu et al., 2005). IRAK-1 phosphorylation contributes to its subsequent ubiquitination or sumoylation. Sumoylated IRAK-1 undergoes cytoplasm to nuclear translocation and participates in Stat3 phosphorylation and transcriptional regulation of selected genes (Huang et al., 2004). On the other hand, IRAK-1 ubiquitination leads to proteosome mediated protein degradation (Yamin and Miller, 1997). The balance of IRAK-1 sumoylation and ubiquitination may therefore regulate both cellular IRAK-1 protein level and its function (Figure 1). Besides mediating TLR signaling, IRAK-1 also participates in
the regulation of adaptive immune response. For example, T cell co-stimulatory molecule CD26 can trigger the association of IRAK-1 with caveolin on antigen presenting monocytes, which is responsible for the subsequent CD86 expression (Ohnuma et al., 2004).

Differential splicing events in human cells give rise to two additional IRAK-1 variants, IRAK-1b and IRAK-1c. IRAK-1b derives from alternative splicing and deletion of 90bp within exon 12, which yields an in-frame deletion of 30 amino acids (residues 514-543) (Jensen and Whitehead, 2001). IRAK-1c is due to alternative splicing and deletion of exon 11 and part of exon 12 (Rao et al., 2005). IRAK-1b exists in minute amount (less than 1% of IRAK-1) in most human cells and tissues with unknown function. On the other hand, the full length IRAK-1 and IRAK-1c are abundantly expressed in human leukocytes and most tissues (Rao et al., 2005; Su et al., 2006).

In contrast to IRAK-1, both IRAK-1b and IRAK-1c are stable and do not undergo covalent modification upon challenge (Bear et al., 2000; Jensen and Whitehead, 2001; Su et al., 2006). Overexpression of IRAK-1c blocks IL-1β induced MAP kinase activation, suggesting that IRAK-1c may serve as a negative regulator of inflammation. Intriguingly, IRAK-1 is absent and IRAK-1c is the predominant form in young human brain tissues (Rao et al., 2005; Su et al., 2006). The absence of full length IRAK-1 may help keeping human brain in an immune-privileged state. In contrast to young humans, we recently found that both IRAK-1 and IRAK-1c are equally present in brain tissues obtained from aged humans. This may bear significant implication in terms of aging. Increased chronic inflammation is a hallmark of aging process as evidenced by local infiltration of inflammatory cells such as macrophages, and higher circulatory levels of pro-inflammatory cytokines, complement components and adhesion molecules. Consequently, aging is often accompanied by increasing incidences of chronic inflammatory diseases such as Alzheimer's or Parkinson's disease. The molecular mechanisms
contributing to the chronic inflammatory state during cellular senescence and aging process is not clearly understood. Our finding that the full length IRAK-1 and IRAK-1c are equally present in aged human brains may provide at least a partial explanation for the aging process. Future studies determining the mechanism of IRAK-1 mRNA differential splicing and the function of different IRAK-1 splice forms are warranted.

Given the roles IRAK-1 play in mediating both the innate and adaptive immune responses, it is not surprising that variation in IRAK-1 gene will lead to diverse inflammatory diseases. Indeed, deletion of IRAK-1 gene in mice decreases the risk of experimental autoimmune encephalomyelitis (EAE) (Deng et al., 2003). We have found that IRAK-1 in leukocytes from atherosclerosis patients is constitutively activated/sumolyted and localizes in cell nucleus (Huang et al., 2004). Furthermore, our human population-based study indicates that genetic variation in IRAK-1 gene correlates with the severity of atherosclerosis and serum C reactive protein levels (Larkoski et al., 2005). There are two IRAK-1 haplotypes and the rare variant haplotype (~10% of human population) contains two exon single nucleotide polymorphisms (SNPs). Humans harboring the variant IRAK-1 gene tend to have higher serum CRP levels and higher risk for diabetes and hypertension (Larkoski et al., 2005). IRAK-1 gene variation is also linked to the risk of sepsis.

Arcaroli et al. recently demonstrated that sepsis patients with the rare variant IRAK-1 haplotype have increased incidence of shock, prolonged requirement for mechanical ventilatory support, and greater 60-day mortality (Arcaroli et al., 2006).
IRAK-2

IRAK-2 was initially identified by Dixit’s group based on the search of the human expressed sequence tag (EST) database for sequences homologous to IRAK-1 (Muzio et al., 1997). Subsequent screening of a human umbilical vein endothelial cell cDNA library resulted in the isolation of a full-length cDNA clone which encodes a 590-amino acid protein with a predicted size of 65 KD. Upon overexpression, IRAK-2 can associate with MyD88 as well as TRAF6, and activate NFκB-dependent reporter gene expression. Intriguingly, IRAK-2, instead of IRAK-1 can also interact with another distinct TLR intracellular adaptor molecule Mal/TIRAP (Schoenemeyer et al., 2005). Dominant negative IRAK-2 can block Mal/TIRAP-induced signaling while dominant negative IRAK-1 fails to do so. These studies suggest that IRAK-2 may selectively be recruited by Mal/TIRAP and participate in NFκB activation. Besides activating NFκB, IRAK-2 also participates in the regulation of cellular apoptosis (Ruckdeschel et al., 2002). Dominant negative IRAK-2 can diminish LPS-induced macrophage apoptosis (Ruckdeschel et al., 2002).

O’Neil’s group has identified the murine IRAK-2 gene, which locates at chromosome 6 at position E3 (Hardy and O’Neill, 2004). In contrast to its human counterpart which only encodes one single transcript, the murine IRAK-2 gene can generate four alternatively spliced isoforms (designated as IRAK-2a, 2b, 2c, and 2d) that have various N-terminal deletions. Upon overexpression, IRAK-2a and IRAK-2b could activate, while IRAK-2c and IRAK-2d inhibit NFκB activation. Alternative splicing of the IRAK-2 gene in mice instead of human reflects the distinct difference in human and murine TLR signaling process and innate immunity regulation. The physiological function of IRAK-2 is poorly defined. Intriguingly, it was reported that several
cases of human liver tumors harbor hepatitis B virus (HBV) DNA insertion near the IRAK-2 gene, which implies that IRAK-2 and related cellular signaling pathways may regulate human carcinogenesis (Paterlini-Brechot et al., 2003). Further studies are needed to determine the biochemical regulation of IRAK-2 and its participation in various cellular signaling pathways.

**IRAK-M**

Using the similar EST search, Wesche *et al.* identified a murine EST sequence which encodes a polypeptide sharing significant homology with IRAK-1 (Knop *et al.*, 1998). Human IRAK-M gene is mapped to chromosome 12 at position 12q14.1-12q15 and its murine homolog is mapped to chromosome 10. Screening of human peripheral blood leukocyte library with this EST sequence resulted in the isolation of a full length cDNA clone that encodes a protein with 596 amino acids and a calculated molecular mass of 68 kDa. Northern blot analysis revealed that IRAK-M transcript is primarily present in the peripheral blood leukocytes and monocytic cell lines. Initial studies revealed that IRAK-M overexpression can activate NFκB activity (Knop *et al.*, 1998). Strikingly, later studies using IRAK-M−/− cells indicate otherwise. IRAK-M−/− macrophages exhibit enhanced NFκB activity and elevated expression of various inflammatory cytokines upon stimulation with several TLR ligands, indicating that IRAK-M may actually inhibit NFκB activation (Kobayashi *et al.*, 2002). Phenotypically, IRAK-M−/− mice develop severe osteoporosis, which is associated with the accelerated differentiation of osteoclasts, an increase in the half-life of osteoclasts, and their activation (Huang *et al.*, 2005). Furthermore, endotoxin tolerance, a phenomenon often observed in leukocytes with prolonged endotoxin treatment or human blood cells from sepsis patients, is significantly reduced in IRAK-M−/− murine macrophages (Kobayashi *et al.*, 2002). IRAK-M levels are elevated in human
monocytes isolated from septic patients, as well as healthy human monocytes and murine macrophages treated with LPS (Escoll et al., 2003; Nakayama et al., 2004). This and other studies indicate that IRAK-M may help to attenuate TLR signaling and prevent excessive inflammation.

**IRAK-4**

Lastly, yet another EST search yielded the human IRAK-4 cDNA sequence that encodes a distinct polypeptide sharing significant homology with the other IRAKs (Hu et al., 2002). Human IRAK-4 gene is mapped to chromosome 12 at position 12p11.22 and its murine homolog can be mapped to chromosome 15. Full-length IRAK-4 cDNA encodes a protein with 460 amino acids and a calculated molecular mass of 52 kDa. In contrast to IRAK-1 or IRAK-M deficient mice, IRAK-4−/− mice exhibit severe impairment in NFκB activation and expression of various inflammatory cytokines upon challenges with several TLR ligands (Suzuki et al., 2002b). Overexpression of kinase-dead IRAK-4 mutant strongly diminishes IL-1/LPS induced NFκB activation, pointing to the essential role of its kinase activity (Hu et al., 2002). MyD88 is critically involved in recruiting IRAK-4 into the TLR4 complex (Hu et al., 2002). These studies indicate that IRAK-4 is the primary kinase in the TLR signaling process essential for mediating NFκB activation. Recently, a study reported that IRAK-4 is also critically involved in T cell receptor (TCR)-induced T cell proliferation through NFκB activation (Suzuki et al., 2006). T cell responses in vivo are significantly impaired in IRAK-4 deficient mice. Upon TCR stimulation, IRAK-4 is recruited to T cell lipid rafts, where it can associate with Zap70 and activate protein kinase C. This finding indicates that there is an intricate connection between innate and adaptive immune system activation, and IRAK-4 may be directly involved in the
cross talk between the two systems.

Because of the central role IRAK-4 plays in mediating NFκB activation and innate immunity signaling, humans carrying IRAK-4 gene variation may be more prone to microbial infections. A study by Picard et al. revealed that IRAK-4 mutations are present in three children suffering from persistent pyogenic bacteria infection and poor inflammatory responses (Picard et al., 2003). These patients do not respond to IL-1β, IL-18, or any of the TLR1-6 or 9 ligands tested, as assessed by activation of NF-κB and p38-MAPK, and induction of IL-1β, IL-6, IL-12, TNFα, and IFN-γ. It is intriguing that the spectrum of infections was relatively narrow, with most infections caused by Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus pneumoniae*. The patients showed poor sign of inflammatory response and the frequency of infection decreased with age, potentially due to the compensatory action of adaptive immunity. In a separate study, a patient was identified who suffered from recurrent bacterial infections and failed to respond to gram-negative LPS *in vivo*, and whose leukocytes were profoundly hyporesponsive to LPS and IL-1 *in vitro* (Medvedev et al., 2005). This patient also exhibits deficient responses in a skin blister model of aseptic inflammation. Cloning and sequencing of IRAK-4 gene revealed that this patient expresses a "compound heterozygous" genotype, with a point mutation (C877T in cDNA) and a two-nucleotide, AC deletion (620-621del in cDNA) encoded by distinct alleles of the IRAK-4 gene. Both mutations encode proteins with an intact death domain, but a truncated kinase domain, therefore precluding expression of full-length IRAK-4. Recently, a case of invasive, systemic, extraintestinal Gram-negative *Shigella* infection was reported in a patient with inherited IRAK-4 deficiency (Cardenes et al., 2006). This case indicates that although the pyogenic Gram-positive bacteria *Staphylococcus* and *Pneumococcus* remain the most frequent pathogens associated with IRAK-4 deficiency,
Gram-negative bacteria such as *Shigella* may threaten humans with IRAK-4 deficiency and cause severe illness and mortality.

**CONCLUSIONS**

In summary, collective research efforts regarding IRAKs studying gene expression, message splicing, protein structure and function, signal transduction, cellular activation of human and murine immune cells, as well as genetic variation in humans have revealed essential roles of IRAK family proteins in both innate and adaptive immune responses. Although IRAK proteins share some common structural features, it is becoming apparent that they are not redundant and each has unique and distinct function. Furthermore, each member may have multiple roles depending upon its modification, cellular and tissue distribution. Besides mediating various TLR signaling pathways, IRAKs are also involved in other signaling networks including T cell receptor and B cell receptor mediated signaling. Conceivably, these molecules pose as viable targets for designing new therapeutic strategies for various human inflammatory diseases.
**FIGURES**

**Table 1. IRAK Family Genes and Related Diseases**

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<tr>
<td>IRAK-2</td>
<td>3p25.3-3p24.1 position E3</td>
<td>Chromosome 6, Not reported</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
<tr>
<td>IRAK-M</td>
<td>12q14.1-12q15</td>
<td>Chromosome 10, Not reported</td>
<td>Not reported</td>
<td>IRAK-M−/− mice develop Osteoporosis (28)</td>
</tr>
<tr>
<td>IRAK-4</td>
<td>12p11.22</td>
<td>Chromosome 15, Point mutation (C877T in mRNA) leading to expression of a truncated protein</td>
<td>Microbial infections (10,32,33)</td>
<td>IRAK-4−/− mice have increased mortality upon bacterial infection (2)</td>
</tr>
</tbody>
</table>
Figure 1 Illustration of IRAK-1 protein regulation and function
2.2 Toll-Like Receptors (TLRs), innate immunity, and inflammation

The innate immune system serves as the first line of defense against pathogens and infection. The main function of innate immunity is to recognize pathogen-associated molecular patterns (PAMPs) and initiate the intracellular downstream signaling pathways to prevent infection and eliminate microbes. TLRs are a family of transmembrane proteins which serve as pattern recognition receptors for a variety of microbe-derived molecules and stimulate innate immune responses to the microbes expressing these molecules. TLRs are composed of an N-terminal leucine-rich-repeat (LRR) for ligand binding, a single transmembrane domain, and an intracellular C-terminal portion which has a Toll/IL-1 receptor (TIR) homology domain (Beutler, 2004; Trinchieri and Sher, 2007). TLRs are widely expressed on the surfaces of different cell types, including monocytes/macrophages, mast cells, B lymphocytes, neutrophils, and dendritic cells, etc. There are thirteen characterized TLRs in mammals; a total of eleven in humans and thirteen in mice (Kawai and Akira, 2006). Much is known about TLRs 1-9 and 11, but the functions of TLR10, 12, and 13 are still unclear. TLRs 1-6, with the exceptions of TLR3, 10, and 11, are located on the plasma membrane of most cells, while TLR3 as well as TLRs 7-9 are located in the endosomal compartments of most cells. TLRs recognize and bind particular ligands, which is referred to as TLR binding specificity. For example, TLR2 cooperates with TLR1 or TLR6 in recognition of triacyl or diacyl lipopeptides, respectively; TLR3 can recognize dsRNA; TLR4 recognizes LPS from Gram negative bacterial cell walls; TLR5 recognizes bacterial flagellin; TLR7/8 recognizes imidazoquinolines and ssRNA; TLR9 recognizes CpG DNA from bacteria and viruses; TLR11 recognizes profiling, and TLR12 function is still unclear (Figure 2).
PAMP binding initiates the TLR signaling pathway. First, TIR-containing adaptors are recruited to the TIR domain of TLRs. Different TLRs may bind to different adaptors. For example, signaling through TLRs 1, 2, 5-9 is mediated by Receptor-associated myeloid differentiation factor 88 (MyD88). Meanwhile, TLR3 exclusively utilizes TRIF for signaling (Botos et al., 2009), and TLR4 utilizes both MyD88 and TRIF. These differences allow for interaction of different molecules in various downstream pathways and confer specificity of the downstream effects. For example, MyD88 interacts with IRAK-4 via death domains. Thus, once IRAK-4 binds MyD88, it recruits tumor necrosis factor receptor-associated factor 6 (TRAF6) to the MyD88/IRAK-4 complex. Then TRAF6 activates transforming growth factor β-activated kinase
1 (TAK1), which then activates the IKKα/β. The end result is the activation of NFκB and the transcription of the innate immune response effectors. Also, MyD88 can recruit IRAK-1 and potential downstream proteins such as TAK1, although this downstream pathway needs further investigation. The MyD88/TRAF5/IRAK-1 complex mediates the activation of interferon regulatory factor 5/7 (IRF-5/7) for cytokine and IFNα production. However, the involvement of IRAK-M, another IRAK family member, still remains unclear (Figure 3). Recent research has found that IRAK-M plays a negative regulation role in intracellular signaling pathway (Kobayashi et al., 2002) and is involved in peptidoglycan-induced tolerance (Nakayama et al., 2004).

Figure 3 Innate immunity intracellular signaling network
2.3 Cell migration and immunity

Cell migration is a complex process that is essential for normal embryonic development and for many important pathological conditions. During the gastrulation process, cells first migrate as sheets to form three layers: endoderm, ectoderm, and mesoderm. Then, cells within these three layers migrate to specific areas throughout the development of the embryo and differentiate into specific tissues and organs. In the adult, cell migration is central to normal states and in maintaining homeostasis. For example, leukocytes migrate from circulation to an area of infection and ingest bacteria, which is important for mounting an immune response. Some homeostatic processes such as wound repair and skin renewal also require cell migration. In addition, aberrant cell migration can contribute to many pathological processes, including vascular diseases, tumor formation and metastasis, and some chronic inflammatory diseases such as rheumatoid arthritis. For example, during the progress of tumor formation, endothelial cells may migrate from blood vessels into the tumor tissue, where they proliferate and contribute to the construction of new vascular networks. Meanwhile, tumor cells can migrate out of the primary site and into the circulation where they can proliferate, and then enter a new location, which is the process of metastasis. Thus, understanding immune cell migration during inflammation is essential in the process of identifying future therapeutic targets.

Cell migration can be thought of as a cyclical process (Ridley et al., 2003). Initially, a cell responds to a migration-promoting agent and begins to polarize and extend a protrusion in the direction of the stimulus. These protrusions include lamellipodia and filopodia, which are driven by actin polymerization. Adhesion complexes then are formed to stabilize the protrusions by attaching to the extracellular matrix (ECM) on which the cell is migrating. These adhesions
provide a means of traction for the cell to pull itself forward, and they are disassembled at the cell rear as the cell retracts, which completes a cell migration cycle. Adhesion complexes can also initiate signals regulating adhesion dynamics and protrusion formation. Most cells, like macrophages, extend their protrusions in one direction, while fibroblasts are more plastic and can extend protrusions in many directions.

A number of proteins are involved in the main three steps of cell migration: cell polarization, cell protrusion and adhesion, and formation of rear retraction. Adhesion complexes are composed of the integrin family of transmembrane receptors and associated structural molecules. Meanwhile, the small GTPases, including Rac and Cdc42, play prominent roles in regulating the entire process. Rac1, one member of the Rac family, induces the formation of small adhesions at the leading edge and controls cell adhesion and motility. Furthermore, microtubules function in the modulation of adhesion disassembly, probably through the regulation of Rac activity.

In vivo, stimulators are always provided by the surrounding environment. Many different molecules can serve as extracellular signaling agents in initiating cell migration. For example, in the case of leukocytes, secreted chemokines can lead to a migratory phenotype and direct movement of these cells by concentration gradient. On the other hand, in vitro culture conditions allow for many cell types to become polarized due to a front and rear asymmetry. The stabilized, directional movement of cells requires persistent external stimulators. Most cells can sense the presence of various chemotactic gradients and migrate in one specific direction. However, fibroblasts in particular are more plastic and can extend protrusions from many positions, so they can change directions. Migratory immune cells include granulocytes (neutrophils, eosinophils, and basophils), monocytes/macrophages and dendritic cells, and lymphocytes (T cells and B
cells) (Luster et al., 2005). Among these different cell migration behaviors, macrophage migration is currently being widely studied.

2.4 Macrophage migration

Macrophages are key components of innate immunity, and are classified as white blood cells. They are found in almost all organs and connective tissues. The formation of macrophages is determined by the migration of blood monocytes into the tissues of the body, where they then differentiate into specific types of macrophages. For example, resident tissue macrophages include Kupffer cells in the liver, microglia in neural tissue, and osteoclasts in bone tissue. Additionally, resident tissues macrophages can secret cytokines that recruit other immune cells from the blood to differentiate into macrophages. Upon stimulation, macrophages can also migrate to a site of infection to remove cellular debris, apoptotic cells, as well as pathogens. Moreover, macrophage migration plays an important role in many diseases such as tumor formation (Condeelis and Pollard, 2006; Murdoch and Lewis, 2005; Wyckoff et al., 2004) and atherosclerosis (Libby, 2002). For example, the formation of atherosclerotic lesions is influenced by the recruitment of macrophages, which in turn engulf oxidized low-density lipoprotein (oxLDL), and respond by producing inflammatory mediators (Glass and Witztum, 2001). Amongst all macrophage phenotypes, bone marrow-derived macrophages (BMDMs) are highly productive, well-defined macrophages expressing all the structure and signaling molecules for migration. Thus, we used BMDM to study macrophage migration.

2.5 Current understanding of regulation of cell migration

Cell migration is a highly integrated process in which many regulators are involved, including
extracellular migration promoting agents, intracellular signaling proteins, and intracellular actin cytoskeleton regulatory molecules.

2.5.1 VASP, an intracellular actin cytoskeleton regulatory molecules

VASP belongs to *Drosophila*-Enabled (Ena)/VASP families which are a conserved family of actin regulatory proteins. They have been implicated in actin-based processes such as fibroblast migration, axon guidance, and T-cell polarization, and are also important for the actin-based motility of the intracellular pathogen *Listeria monocytogenes* (Krause et al., 2003; Kwiatkowski et al., 2003). VASP plays an important role in *Listeria* internalization (Bierne et al., 2005) and participates in cell adhesion, the formation of filopodia, and chemotaxis in *Dictyostelium* (Han et al., 2002). VASP protein has an N-terminal *Drosophila* enabled vasodilator-stimulated phosphoprotein homology-1 (EVH1) domains, a C-terminal EVH2 domain, and a proline-rich domain in the middle. The EVH2 domain is in charge of actin binding and tetramerization while the proline-rich domain interacts with the SRC homology3 domain (SH3). Meanwhile, the WW domain is involved in profilin binding and the EVH1 domain is a 115 residue domain involved in protein-protein interaction, linking host proteins to various signal transduction pathways (Sato et al., 2005). Many EVH1-containing proteins are associated closely with actin-based structures and are also involved in reorganization of the actin cytoskeleton (Ball et al., 2002). Members of the Ena/VASP family are key players in regulating actin filament assembly through their association with binding partners. Ena/VASP proteins interact with these partners via the highly conserved EVH1 domain. It has been determined that the EVH1 domain within Ena/VASP family members can specifically bind to a motif (F/W/L/Y) PPPP (Renfranz and Beckerle, 2002). EVH1-binding motifs have also been found in cytoskeleton proteins such as zyxin and vinculin, as well as in the *Listeria* protein ActA. Proteins with ActA repeats are potential binding partners.
for Ena/VASP proteins. Only a handful of potential EVH1 domain-binding partners have been characterized thus far and almost all of them are structure protein. No signaling protein has been identified to bind to EVH1 domain of VASP. Interestingly, after careful scanning of the IRAK-1 sequence, we found that IRAK-1 has a single LWPPP motif between the N-terminal death domain and the central kinase domain (Figure 4); thus it could theoretically serve as a docking site for an EVH1 domain. With this information, we can provide a hypothesis: IRAK-1 may interact with the VASP EVH1 domain by its LWPPP motif, which may lead to a change in macrophage migration ability.

Figure 4 IRAK-1 protein sequences

| MAGGPGPGEF AAPGAQFLYY EVPPWVMCRF YKVMDALEPA DWCQFAALIV RDQTELRLCE RSGQRTASVL WFWINRANV ADLVHILTHL QLLRADITI AWHPAPLPS PGTTARPSS IPAPAEAEAW SPRKLPSAS TFLSPAFPGS QTHGSFELGL VPSPA LWPPP PP SPAPSSTK PGPESVSSL QGARPSPFCW PLCIESRGTH NFSEELKIGE GGFCVYARV MRNTVYAVKR LKENADLEWT AVKQSFLETV EQLSRFRHPN IVDFAGYCAQ NGFYCLVYGFL LPNGSLEDRL HCQTACPPPL SWPQRLDILL GTARAIQFLH QDSPSLIHGD IKSSNVLLDE RLTFLKGDGF LARFSGRAGS SPSQQSMVAR TQTVRGTIAY LPEEYIKTGR LVDTDTFSF GVVLETLAG QRAVKTHGAR TKYKDLVVE EAAEAGVALR STQSTLQAGL AADAWAAPIA MQYIKKHLDP RPGPCPELGG LGLGQACCC LHRRAKRPPP MTQVYERLEK LQAVVAGVPG HLEAASCIPP SPQENSYVSS TGRAHSGAAP WQPLAAPSQA SAQAAEQLQR GPNQPVESDE SLGLSAALR SWHLTPSACL DPAFLREAGC PQGDTAGESS WGSGPGSRPT AVEGLALGSS ASSSSEPPQI INPARQKMV QKLALYEDGA LDSQLLSSS SLPGRLQEQD RQGPEESDEF QS |

2.5.2 Rho GTPases, intracellular actin cytoskeleton regulatory molecules

The Rho family proteins, Rac and Rho, are small GTP-binding proteins that are well known to regulate cell migration in many different cell types. They are believed to be key regulators of cell migration through their effects on the cytoskeleton and cell adhesion. Rho regulates the contraction and retraction forces required in the cell body and at the cell rear, while Rac
regulates actin polymerization and membrane protrusion at the front of the cell. Other investigators have determined that Rac and PI3-kinase (PI3K) interact directly with each other during cell migration and that Rac activation can stimulate PI3K, leading to the production of PI (3, 4, 5) P3, which serves as positive feedback (Raftopoulou and Hall, 2004).

The Rac family consists of 20 genes in mammals (Boureux et al., 2007), where there are three Rac isoforms: Rac1, Rac2 and Rac3. These isoforms are highly homologous, differing only significantly in their C-terminal domains (Wennerberg and Der, 2004). Rac proteins are believed to be important for cell migration by regulating the formation of lamellipodia, which are generally found at the leading edge of migrating cells, as well as by promoting the formation of integrin-based adhesions (Ridley et al., 2003). Rac proteins may contribute to cell migration via several downstream targets. For example, neutrophils and hematopoietic stem/progenitor cells derived from Rac1/2- null mice are severely impaired in their ability to migrate (Gu et al., 2003). Rac1 is also involved in macrophage migration through its regulation of ERK1/2 phosphorylation (Bhavsar et al., 2009). Additionally, cytoskeletal dynamics are regulated by Src-family tyrosine kinases (SFKs) and c-Abl. Previous studies using selective inhibitors and cells from SFK-deficient mice demonstrated that SFK and c-Abl can regulate the activation of the small GTPases Cdc42 and Rac in murine macrophages (Baruzzi et al.). Previous studies have identified the cross-talk between VASP and Rac1 (Schlegel and Waschke, 2009; Zhang et al., 2009), and between MCP-1 and Rac1 (Lopes et al., 2002; van Golen et al., 2008). In addition, O’Neill’s group was the first to report that dominant negative form of IRAK-1 antagonizes the function of Rac1, by using dominant negative constructs and plasmid transfection assays (O'Neill, 2000).
2.5.3 Chemokines, extracellular migration promoting agents

Chemokines are small secreted polypeptides (60-100 amino acids), which constitute a large family of chemotactic cytokines whose main function is to regulate cell trafficking and the effector functions of leukocytes (Mackay, 2001). Thus, chemokines play an important role in inflammation and host defense against pathogens. Based on the number and location of the cysteine residues at the N-terminus, chemokines can be classified into four subfamilies: CXC, CC, CX3C, and XC, which is in agreement with the systematic nomenclature (Rollins, 1997). Chemokines are secreted in response to signals such as pro-inflammatory cytokines, where they play an important role in recruiting monocytes, neutrophils and/or lymphocytes. Chemokines initiate chemotaxis through the activation of G-protein-coupled receptors (GPCRs) and the involvement of adhesion molecules and glycosaminoglycans (GAGs) (Hyduk et al., 2007). Chemokines bind to transmembrane receptors and contribute to the activation of downstream intracellular signaling cascades, thereby promoting cell migration towards the chemokines. The chemotactic functions of chemokines are also cell-specific. For example, MCP-1 is chemotactic for monocytes and macrophages. Similarly, chemokines can also be divided into two main subfamilies based upon the physiologic and pathologic function of the cell migration: homeostatic and inflammatory. The homeostatic group of chemokines are produced in low concentrations and fulfill housekeeping functions, while the inflammatory chemokines are abundant at sites of inflammation and injury, and function to recruit leukocytes (Loetscher and Moser, 2002; Moser and Willimann, 2004). TLR ligands such as LPS act as activators of the immune response through the induction of chemokines, which in turn serve as the secondary mediators to attract additional inflammatory cells (Gouwy et al., 2009; Walton et al., 2009; Zoico et al., 2009).
MCP-1 is a member of the CC chemokine family and is one of the key factors involved in the initiation of inflammation. MCP-1 is located on chromosome 17 (chr.17, q11.2) and was the first CC chemokine discovered in humans. There are four members in the MCP family: MCP-1, 2, 3, and 4. MCP-1 is the most widely studied member among these four members. MCP-1 triggers chemotaxis and transendothelial migration of monocytes and macrophages to inflammatory sites by interacting with the membrane CC chemokine receptor 2 (CCR2). MCP-1 can be produced by various cell types and its elevated expression is closely related to the presence of many diseases, such as cardiovascular disease (Niu and Kolattukudy, 2009), atherosclerosis (Chen et al.; Tian et al., 2009), viral arthritides (Suhrbier and Mahalingam, 2009), acute coronary syndromes (Gonzalez-Quesada and Frangogiannis, 2009), chronic obstructive pulmonary disease (Murugan and Peck, 2009), and diabetes (Brosius et al.; Giunti et al.; Yuasa et al., 2009).

2.5.4 Other regulators of cell migration

Since cell migration is a highly integrated and complex process, we must acknowledge that there are many other complex mechanisms involved in the regulation of cell migration. Integrins are also essential for cell migration and invasion because they directly mediate adhesion to the extracellular matrix, as well as the intracellular signaling pathways that control cytoskeletal organization. Thus, over the past several years, research has led to the development of integrin and protease inhibitors that are now being tested in clinical trials (Hood and Cheresh, 2002). In addition, integrin function can be regulated by changes in glycosylation. However, the role of integrin glycosylation in migration still remains unclear (Janik et al.).

Furthermore, Macrophage migration inhibitory factor (MIF) plays a central role as a regulator of innate immune and inflammatory responses. It upregulates TLR4 expression and also activates
the ERK1/2 signaling pathway (Calandra and Roger, 2003). In addition, MIF contributes to tumor cell migration and many aspects of tumor progression (Ren et al., 2003).
2.6 LITERATURES CITED


CHAPTER 3: SPECIFIC AIMS

3.1 Specific Aim I: To study the regulation of IRAK-1 on VASP, an intracellular actin regulatory protein

Our first research aim was to study the function of IRAK-1 on actin regulatory protein during macrophage migration. Since cell migration is a multifaceted procedure, in which many steps and many proteins are involved, it is difficult to identify the specific substrate for IRAK during this complex process. However, VASP has an EVH1 domain which has been proven to bind to (F/W/L/Y) PPPP motifs, and IRAK-1 protein possesses such a motif: LWPPPP. Consequently, it is likely that IRAK-1 directly interacts with VASP and thus regulates downstream macrophage migration. Since VASP is a substrate for PKC and PKCε is an isoform of the PKC family which is highly expressed in murine macrophages, we chose to use PMA, a potent inducer of PKCε, VASP phosphorylation, and subsequent cell migration, for our research. Figure 1 gives an illustration of a potential pathway for PMA-induced macrophage migration in which IRAK-1 is involved, as well as all of the experimental techniques utilized.

i. To investigate whether IRAK-1 interacts with VASP, two different techniques were utilized. First, co-immunoprecipitation (co-IP) was implemented to test the binding of VASP in an IRAK-1 IP complex and IRAK-1 in a VASP IP complex. Following this, immunohistochemical staining was performed with FITC-combined IRAK-1 antibody and Alex red-combined VASP antibody. Cells were then observed and recorded using a confocal microscope.

ii. To better define the structural requirement for this binding, Flag-tagged WT and mutated IRAK-1 plasmids were used for transfection and IP assay.
iii. To study IRAK-1 function on PMA-induced VASP phosphorylation, BMDMs from WT and IRAK-1−/− mice were stimulated with PMA at specific times and VASP phosphorylation was analyzed using SDS-PAGE gels.

iv. To test the association between IRAK-1 and PKCε, co-IP was again utilized to identify whether IRAK-1 is bound in the PKCε immunoprecipitate. In addition, a peptide-specific PKCε inhibitor was added to confirm PKCε function on VASP phosphorylation.

v. After these molecular mechanism studies, assays on biological phenomena, specifically cell migration, were performed using BMDMs from WT and IRAK-1−/− mice. There are many well-established assays to study cell migration, including the Boyden chamber assay, wound-healing assay, Dunn chemotaxis chamber and time-lapse microscopy, and cell-spreading assays. The trans-well cell migration assay, also known as the modified Boyden chamber assay, is ideal for the quantitative analysis of leukocyte migratory responses. It is based on an insert with a microporous membrane separating two
median-filled compartments. In general, cells are placed in the upper compartment and migrate through the pores on the membrane into the lower compartment, in which chemotactic agents are added. After an appropriate incubation, the membrane is fixed and stained and the number of migrated cells is determined under microscope (Figure 2).

![Flow chart of Trans-well migration assay](image)

**Figure 2: Flow chart of Trans-well migration assay**

vi. Draw a schematic illustration to link IRAK-1 with signaling molecules and cytoskeleton-related proteins involved in macrophage migration.

### 3.2 Specific Aim II: To study the regulation of IRAK-1 on MCP-1, an extracellular stimulator of macrophage migration.

Chemokines are a large family of cytokines related to cell recruiting and trafficking. Our next research aim was to study the molecular mechanism of IRAK-1 function during chemokine-related macrophage migration. Since there are so many unique members of the chemokine family, we first decided on a chemokine as our research target. After careful literature
searching, we chose to examine MCP-1, a widely studied chemokine involved in inflammatory diseases. Furthermore, other researchers have found that MCP-1 is induced by LPS, a potent activator of the TLR4 pathway in which IRAK-1 is involved. To examine the involvement of IRAK-1 during MCP-1-related macrophage migration, we used LPS as the stimulant to induce cell migration and compare the migration ability of BMDMs from WT and IRAK-1⁻/⁻ mice. Here, we give an illustration of a possible pathway for LPS-induced cell migration in which IRAK-1 is involved and all the techniques used to identify this pathway (Figure 3).

Figure 3: Illustration of experiment design for Specific Aim II

i. To test whether IRAK-1 plays a role in LPS-induced MCP-1 production. Supernatants from LPS-stimulated BMDMs from WT and IRAK-1⁻/⁻ mice and blood plasma samples from WT and IRAK-1⁻/⁻ mice after LPS injection or from Apoe⁻/⁻ and Apoe⁻/⁻/IRAK-1⁻/⁻
mice after three months of high-fat diet feeding were collected to measure the MCP-1 protein concentration by multi-plex assay.

ii. To determine the effect of LPS on MCP-1 expression \textit{in vitro} and \textit{in vivo}, mRNA samples from BMDMs from WT and IRAK-1\(^{-/-}\) mice and splenocytes from Apoe\(^{-/-}\) and Apoe\(^{-/-}/\text{IRAK-1}^{-/-}\) mice after three months of high fat diet feeding were harvested and measured by real-time RT-PCR.

iii. To further study the molecular mechanism of IRAK-1 on MCP-1 gene transcription. Firstly, WT and IRAK1\(^{-/-}\) BMDM cells were treated with LPS. Whole cell lysates and nuclear extracts were resolved on SDS-PAGE gels and blotted with antibodies specific for C/EBP\(\delta\) and NF\(\kappa B\). Secondly, BMDMs induced with LPS were subjected to chromatin immunoprecipitation (ChIP) assays using the indicated antibodies and primers specific to the proximal promoter of murine MCP-1.

iv. To study the potential association between IRAK-1 and IKK\(\varepsilon\), co-IP was then applied to test the presence of IRAK-1 in the IKK\(\varepsilon\) immunoprecipitates. In addition, Flag-tagged IRAK-1 mutations were used again for transfection and co-IP.

v. To further confirm that IRAK-1 is closely related to IKK\(\varepsilon\)-mediated expression and activation of both C/EBP-\(\delta\) and MCP-1. An IKK\(\varepsilon\) specific inhibitor and IKK\(\varepsilon\) siRNA transfection were performed to remove the IKK\(\varepsilon\) effect. MCP-1 expression was measured by real-time RT-PCR. In addition, western blots were repeated after adding IKK\(\varepsilon\) specific inhibitors.

vi. To confirm IRAK-1 function on LPS-induced murine BMDMs migration. Trans-well cell migration assays were employed to compare migration ability between BMDMs from WT and IRAK-1\(^{-/-}\) mice after LPS treatment.
vii. Draw a schematic illustration to link IRAK-1 with receptors, signaling molecules, and downstream chemokines involved in macrophage migration.

3.3 Specific Aim III: To study the regulation of IRAK-1 on Rac1, an intracellular actin regulatory protein.

Previous studies have determined the presence of cross-talk between VASP and Rac1, as well as between MCP-1 and Rac1. In addition, dominant negative IRAK-1 antagonizes the function of Rac1. These suggest that there is potential interaction between IRAK-1 and Rac1. Since Rac1 is one of the essential regulatory proteins in the cell migration cycle, finding the association between IRAK-1 and Rac1 will provide a more useful guideline for studying cell migration mechanisms. Thus, we examined the relationship between IRAK-1 and Rac1 and will set the specific aims as follows:

i. To investigate whether IRAK-1 interacts with Rac1, co-IP assays were applied to test the binding of Rac1 with IRAK-1.

ii. To identify the binding site between Rac1 and IRAK-1. A co-IP was repeated following the transfection of multiple Flag-tagged IRAK-1 mutation plasmids into Hela cells.

iii. Further experiments are needed to devise a more detailed pathway.
CHAPTER 4:

Interleukin-1 Receptor-associated Kinase-1 (IRAK-1) functionally Associates with PKCε and VASP in the Regulation of Macrophage Migration

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Running title: IRAK-1 regulates macrophage migration

Key words: VASP, IRAK-1, cell migration

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Figure count: 5
Abstract

Macrophage migration is mediated by complex cellular signaling processes and cytoskeleton re-arrangement. In particular, recent advances indicate that the innate immunity signaling process plays a key role in the regulation of macrophage migration. In this report, we have provided evidence demonstrating the involvement of a key innate immunity signaling kinase, Interleukin-1 Receptor-Associated Kinase-1 (IRAK-1) as a critical modulator of macrophage migration. Macrophage migration induced by phorbol 12-myristate 13-acetate (PMA) is significantly attenuated in IRAK-1\(^{-/-}\) macrophages as compared to wild type macrophages. Mechanistically, we demonstrated that IRAK-1 works downstream of PKC\(\varepsilon\) and upstream of VASP, a member of Ena/VASP family proteins. IRAK-1 forms a close complex with PKC\(\varepsilon\) as well as VASP, and participates in PMA-induced phosphorylation of VASP. Notably, IRAK-1 contains a novel EVH1 domain binding motif (L\(^{167}\)WPPP) within its N-terminus, which is responsible for its interaction with VASP. The mutant IRAK-1 (L167A/W168A) fails to associate with VASP. Our findings provide a novel facet regarding the molecular signaling process regulating macrophage migration.
INTRODUCTION

Macrophage migration is regulated at multiple levels by diverse molecules including cellular receptors, intracellular signaling proteins, and cytoskeletal structural molecules (Raftopoulou and Hall, 2004; Ridley et al., 2003; Streuli and Akhtar, 2009). The regulated expressions of cytokines, chemokines, as well as chemokine receptors during the immune cell activation and migration have been extensively studied in the past (Schilling et al., 2009; van der Meulen et al., 2009). However, relatively little information is available regarding the regulation of structural proteins by innate immunity signaling processes.

VASP (Vasodilator-Stimulated Phosphoprotein) is one of the key structural proteins involved in the regulation of cell migration because it is responsible for facilitating cellular focal adhesion, as well as actin filament binding and polymerization (Krause et al., 2003). VASP contains a novel EVH1 domain at its N-terminus that binds with protein partners containing a rare L (W/F) PPPPS/T motif (Peterson and Volkman, 2009). Almost all of the currently identified proteins containing such a motif are structural proteins including zyxin and vanculin (Renfranz and Beckerle, 2002). However, no regulatory proteins (e.g. protein kinases or phosphatases) have been identified that bind directly with VASP.

The Interleukin-1 Receptor Associate Kinase 1 (IRAK-1) is a critical protein kinase involved in innate immunity signaling processes (Gan and Li, 2006; Huang et al., 2005; Ringwood and Li, 2008). IRAK-1 is known to be not only involved in the inducible expression of pro-inflammatory mediators (Bannerman et al., 2002; Hu et al., 2002), but also in macrophage migration. IRAK-1 deficient mice have decreased monocyte /macrophage infiltration to inflamed brain tissues in the EAE model (Deng et al., 2003). However, the molecular
mechanism underlying IRAK-1 mediated macrophage migration is not clearly understood. Intriguingly, we noticed that IRAK-1 contains a perfect match of the LWPPPPSP motif within the highly Pro-Ser rich region, raising the potential prospect that IRAK-1 may serve as a direct regulatory kinase involved in VASP phosphorylation and function. Intriguingly, phosphorylation of VASP at Serine 157 has been well identified to be necessary for agonist-induced cell migration (Blume et al., 2007; Eckert and Jones, 2007). In particular, PMA-mediated activation of protein kinase C (PKC) is known to contribute to VASP phosphorylation at Ser157 (Chitaley et al., 2004; Wentworth et al., 2006). Recent study also revealed that IRAK-1 is associated and regulated by various isoforms of PKCs (Cuschieri et al., 2004; Mamidipudi et al., 2004).

In this report, we tested the hypothesis that IRAK-1 may be a close interacting partner with VASP. Furthermore, we tested the functional relationship between IRAK-1, PKC, and VASP phosphorylation, as well as its implication in macrophage migration.
MATERIALS AND METHODS

Mice and cells

Wild type (WT) C57BL/6 mice were purchased from the Charles River laboratory. IRAK-1\(^{-/-}\) mice on a C57BL/6 background were kindly provided by Dr. James Thomas from the University of Texas Southwestern Medical School. All mice were housed and bred at Derring Hall animal facility in compliance with approved Animal Care and Use Committee protocols at Virginia Polytechnic Institute and State University. THP-1 cells, an undifferentiated human pro-monoecytic cell line, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Bone marrow derived macrophages (BMDMs) were isolated from WT and IRAK-1\(^{-/-}\) mice and maintained as described (Su et al., 2007b). MAT-2 cells, obtained from Dr. Fabio Re, were Hela cells stably transfected with TLR2 and were maintained as described (Su et al., 2007b).

Plasmids and transfection

The wild type pFlag-IRAK-1 plasmid and the C-terminal deletion pFlag-IRAK-1ΔC plasmids were made as described (Su et al., 2007b). The pFlag-IRAK1-L167A/W168A was generated using the GeneEditor site-directed mutagenesis kit (Promega, WI). All constructs were confirmed by automated sequencing. Plasmids were transiently transfected into MAT-2 cells using the lipofectamine 2000 as described by the manufacturer (Invitrogen).

Co-immunoprecipitation and western blot

Cells were washed in phosphate-buffered saline and subsequently lysed with lysis buffer (50 mM
HEPES, pH 7.6, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 20 mM β-glycerophosphate, 1 mM NaF, 5 mM p-nitrophenylphosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 100×protease inhibitors). 800 μg of cell extract was incubated for 1 h with either anti-IRAK-1, anti-VASP (Santa Cruz) or control IgG (Santa Cruz) antibodies followed by overnight incubation with 60 μl of Protein A/G Plus agarose (Sigma). The samples were separated on 10% SDS-polyacrylamide gels and then Western blotted with anti-PKCε (Cell signaling), anti-IRAK-1 or anti-VASP antibodies. Horseradish peroxidase conjugated secondary antibody was then added and the blot was developed employing ECL (Amersham Biosciences).

**Trans-well cell migration assays**

Trans-well migration assays were performed using 8-μm-pore-size trans-well inserts according to the manufacturer's instructions (BD Biosciences). Briefly, BMDMs (4 × 10<sup>5</sup>) suspended in serum-free DMEM medium were added to the top chambers of trans-well inserts in 24-well plate. Stimulators were added to the lower chamber which contains DMEM/1% FBS. Cells were incubated for a proper amount of time, fixed, and then stained with Giemsa solution (Sigma). The numbers of migrated cells present were counted under microscope and expressed as means ± standard deviations.

**Statistical analysis**

Statistical significance was determined using the unpaired 2-tailed Student’s <i>t</i>-test and P-values less than 0.05 were considered statistically significant.
RESULTS

IRAK-1 physically associates with VASP in vivo

Given the presence of a novel EVH1-binding LWPPPSP motif within IRAK-1, we tested whether IRAK-1 may form a close complex with VASP. Human monocytic THP1- cells were used to perform co-immunoprecipitation studies using anti-VASP antibody. As shown in Fig. 1A, IRAK-1 was detected in the VASP immuno-complex. To further confirm their interaction, we performed immunoprecipitation using an anti-IRAK-1 antibody. As shown in figure 1B, VASP was detected in the IRAK-1 co-immunoprecipitates in THP-1 cells. As confirmatory evidence, we performed immunofluorescence staining analyses of murine macrophages, and detected sub-cellular co-localization of IRAK-1 and VASP near the periphery of macrophages (data not shown).

The LWPPP motif within IRAK-1 is required for its interaction with VASP

To better define the structural requirement for their interaction, we have generated various IRAK-1 mutant plasmids. Specifically, we have designed plasmids encoding the full length IRAK-1 (pFlag-IRAK-1), IRAK-1 with C-terminal truncation (pFlag-IRAK-1ΔC), or IRAK-1 containing a point mutation at the LWPPP motif, pFlag-IRAK-1(L167A/W168A) (Fig. 2B). These plasmids were transiently transfected into MAT-2 cells, and all transfected constructs were similarly expressed, as confirmed by Western blot (Fig. 2A). Equal amounts of cell lysates from transfected cells were used to perform co-immunoprecipitation assays using anti-VASP antibody. As shown in Fig.2A, pFlag-IRAK-1 and pFlag-IRAK-1ΔC co-immunoprecipitated with VASP. By marked contrast, the mutant pFlag-IRAK-1(L167A/W168A) was not found in
the VASP co-immunoprecipitates. These results suggest that VASP binding may be mediated by the LWPPPP site located between the death domain and the kinase domain of IRAK-1.

**IRAK-1 and PKCε facilitates PMA-induced VASP phosphorylation at Ser157**

Since IRAK-1 has been shown to associate with PKC, and PKC has been implicated in VASP phosphorylation at serine 157, we subsequently tested whether IRAK-1 is involved in PMA-induced VASP phosphorylation. BMDMs from WT or IRAK-1 deficient mice were treated with PMA for various time periods, and the phosphorylation status of VASP was evaluated by Western blot. As shown in Figure 3, PMA treatment induced a rapid occurrence of an upper phosphorylated VASP band in WT BMDMs. In contrast, PMA-induced VASP phosphorylation was significantly reduced in IRAK-1−/− BMDMs.

We further examined the VASP phosphorylation using a VASP-pSer157 antibody. As shown in Fig. 3, there was a rapid induction of VASP phosphorylation at serine 157 in WT BMDMs. In contrast, there was a ~70% reduction of serine 157 phosphorylation in IRAK-1−/− BMDMs.

We also tested the interaction between IRAK-1 and PKCε, a known PKC isoform involved in the phosphorylation of VASP and cell migration (Stensman and Larsson, 2008). As shown in Fig. 4A, IRAK-1 was co-precipitated with the endogenous PKCε in WT BMDMs.

To confirm that PMA-induced VASP phosphorylation was dependent on PKCε activation, a PKCε-specific inhibitor, myristoylated PKCεV1-2 peptide (BIOMOL), was utilized. As shown in Fig. 4B, PMA-induced VASP phosphorylation was completely abrogated by the addition of the inhibitor peptide.
Taken together, these results indicate that both IRAK-1 and PKCε functionally associate with each other in mediating PMA-induced VASP phosphorylation.

**IRAK-1 participates in PMA-induced trans-swell migration of macrophages**

To test whether IRAK-1 is required for PMA-induced macrophage migration, we performed trans-well migration assays using WT and IRAK-1<sup>−/−</sup> BMDMs. As shown in Figure 5, PMA treatment induced robust migration of WT BMDMs especially at 40nM. In contrast, there was a 70% reduction in cell migration with IRAK-1<sup>−/−</sup> BMDMs.
DISCUSSION

In this study, we demonstrated that IRAK-1 is a novel regulatory kinase closely associated with VASP and responsible for macrophage migration. First, we documented that IRAK-1 interacts with VASP via the LWPPPP motif within IRAK-1. Second, IRAK-1 coordinates with PKC and participates in PMA-induced VASP phosphorylation. Third, IRAK-1 is involved in PMA-induced macrophage migration.

The LWPPPPSP motif is located within the variable region, a region which is uniquely present in IRAK-1 molecule, as it is absent in all other forms of the IRAK family members (IRAK-2, M, and 4). All family members of IRAK-1 share a conserved N-terminal death domain and a kinase domain (Li et al., 2001). In sharp contrast, there is a novel variable region between the death domain and the kinase domain present only in IRAK-1 molecule. Despite the known presence of this domain, no biochemical or physiological function has been assigned to this portion of IRAK-1 molecule. Our study showed a requirement for this novel motif in the interaction of IRAK-1 with a cytoplasm structural protein, VASP. However, the molecular mechanism underlying the requirement for their interaction still requires further study. The LWPPPP motif may either directly participate in the interaction with VASP, or indirectly contribute via altering the secondary structure of other related domains involved in their interaction.

Functionally, our study reveals a glimpse of the dynamic and complex interplay between PKCɛ, IRAK-1 and VASP phosphorylation. PMA is the potent and specific activator of PKC. Given our finding that PMA-induced VASP phosphorylation is significantly attenuated in IRAK-1⁻/⁻ cells, we reason that IRAK-1 works downstream of PKCɛ, and contributes to the
phosphorylation of VASP. On the other hand, blocking of PKCε with a selective inhibitor completely ablates PMA-induced VASP phosphorylation. This indicates that PKC may also directly contribute to VASP phosphorylation. Alternatively, IRAK-1 may serve as a positive feedback regulator, further inducing the activation of PKCε. Consistent with this scenario, a recent report indicates that TLR agonists can induce the activation of PKCε through a MyD88-p38 dependent pathway (Saurin et al., 2008). IRAK-1 is known an activator downstream of MyD88 and is upstream of p38 activation. Conceivably, IRAK-1 activation may lead to p38 activation and PKC phosphorylation and activation. In either of the above two scenarios, deletion of IRAK-1 would attenuate, but not completely ablate, PMA-induced activation of PKC and VASP phosphorylation. Although our data clearly demonstrate the involvement of IRAK-1 in the phosphorylation of VASP, we can not exclude the possibility that IRAK-1 may indirectly contribute to the phosphorylation of VASP. For example, PKC or other related and un-identified enzymes may be recruited to the complex due to IRAK-1, and responsible for VASP phosphorylation.

Our study reveals the pleiotropic nature of IRAK-1 function. IRAK-1 is known to be involved in cellular signaling networks controlling the activation of multiple transcription factors (e.g. NFκB (Song et al., 2006), IRF5/7 (Oganesyan et al., 2006; Schoenemeyer et al., 2005; Uematsu et al., 2005), NFAT (Wang et al., 2008), and nuclear receptor transcription factors (Maitra et al., 2009a; Maitra et al., 2009b)). In addition, IRAK-1 is present in both cellular cytoplasm and nucleus (Su et al., 2007a). How IRAK-1 is capable of performing diverse functions is not clearly understood. Conceivably, the multiple functional domains of IRAK-1, including its kinase domain, an N-terminal death domain, a variable region containing the LWPPPPP motif, and a C-terminal Serine-rich region, may help explain its participation in diverse cellular functions.
Further biochemical and physiological studies are needed to clarify this question.

ACKNOWLEDGEMENTS:

This work is partially supported by funding from National Institute of Health.
FIGURE LEGENDS

Figure 1 IRAK-1 interacts with VASP. (A) Human monocytic THP1- cells were either untreated or treated with 40nM PMA for 15 min. Equal amounts of total cell lysates were harvested and used to perform immunoprecipitation analyses using an anti-VASP antibody. Co-immunoprecipitated protein complexes were resolved on a SDS-PAGE, and blotted with an anti-IRAK-1 antibody. An isotype control IgG antibody was used to perform similar immunoprecipitation studies, which gave no specific signal near the IRAK-1 region. The levels of VASP in the cell lysates are shown in the bottom panel. (B) Extracts from above-treated cells were immunoprecipitated with anti-IRAK-1 antibody. Co-immunoprecipitated protein complexes were resolved on a SDS-PAGE, and blotted with either an anti-VASP or an anti-IRAK-1 antibody. A control IgG antibody was used to demonstrate no non-specific interaction within the region of interest.

Figure 2 Identification of the IRAK-1 motif is necessary for its interaction with VASP. (A) MAT-2 cells were transiently transfected with either pFlag-IRAK-1, pFlag-IRAK-1ΔC, or pFlag-IRAK-1(L167A/W168A) mutants. Equal amounts of lysate were harvested from the transfected cells and used to perform immunoprecipitation analyses using anti-VASP antibody. Co-immunoprecipitated protein complexes were resolved on SDS-PAGE and blotted with an anti-FLAG antibody (top right panel). A control anti-goat IgG was used to perform a similar immunoprecipitation study, and did not give a specific signal near the region of interest (data not shown). The expression levels of various Flag-IRAK-1 mutants within the cell lysates are indicated in the upper left panels and the levels of VASP in the cell lysates are shown in the bottom panels. (B) A diagrammatic illustration of various Flag tagged IRAK-1 full-length and
deletion constructs used in the transfection studies.

Figure 3 IRAK-1 is involved in PMA-induced VASP phosphorylation. (A) BMDMs from WT and IRAK1-/- BMDM mice were treated with 40nM PMA for the indicated time periods. Equal amounts of total cell lysates were resolved on SDS-PAGE and blotted with antibodies specific for the VASP-pSer157, total VASP, or GAPDH as indicated.

Figure 4 IRAK-1 interacts with PKCε. (A) BMDMs from WT mice were either untreated or treated with 40nM PMA for 15 min. Equal amounts of total cell lysates were harvested and used to perform immunoprecipitation analyses using an anti-IRAK-1 antibody. Co-immunoprecipitated protein complexes were resolved on a SDS-PAGE, and blotted with an anti-PKCε antibody. A control anti-rabbit IgG antibody was used to perform similar immunoprecipitation study, which gave no specific signal near the PKCε region. The levels of IRAK-1 in the cell lysates are shown in the bottom panel. (B) BMDMs from WT mice were treated with 40nM PMA for different time course. Specific PKCε inhibitors were added as shown. Anti-VASP-pSer157 and anti-VASP antibody were used as shown.

Figure 5 Depletion of IRAK-1 impairs cell migration. BMDMs from WT and IRAK1-/- mice were loaded onto the top wells of the trans-well chamber without or with PMA added to the bottom chamber at concentration of 4nM, 40nM or 400nM. After incubation in a humidified 5% CO2 incubator for 16 h, the cells on the lower surface of the filter were visualized with microscopy after staining with Giemsa solution. The transmigrated cells were photographed in triplicate wells at 20× magnification (B). Cell migration was determined as the number of cells that had migrated across the filter in five photographed areas (A). Results shown are mean values and error bars represent ± SEM. **, p < 0.01. These findings are similar to those obtained in
two other independent experiments.
FIGURES

Figure 1

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Figure 2

(A) | Lysates | IP VASP |
---|---|---|
Wild Type | W163A/P170A | ΔC | Wild Type | W163A/P170A | ΔC |

IB FLAG

IB VASP

(B)  

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Flag IRAK-1 | 1 | 100 | 220 | 547 | 712 |
Flag IRAK-1 | 1-197 | 100 | 220 | 547 | |
Flag IRAK-1 | 1-197 | 1 | 100 | 220 | 547 | 712 |

W163A/P170A
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Figure 4

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IP IRAK-1

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Figure 5

(A) 

Number of migrating BMDM

WT

IRAK-1-/-

PMA control 4nM 40nM 400nM

(B) 

WT

IRAK-1-/-

Control

4nM

40nM

400nM
REFERENCES


CHAPTER 5

Interleukin-1 Receptor-Associated Kinase-1 (IRAK-1) functionally associates with IKKe and induces MCP-1 Transcription in the Regulation of Macrophage Migration

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Running title: IRAK-1 regulates macrophage migration

Key words: IRAK, MCP-1, IKKe, cell migration, C/EBPδ

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Figure count: 7
ABSTRACT

Macrophage migration is mediated by complex cellular signaling processes and by cytoskeletal rearrangement. In particular, recent advances indicate that the innate immune signaling process plays a key role in the regulation of macrophage migration. In this report, we demonstrated the involvement of a key innate immune signaling kinase, Interleukin-1 Receptor-Associated Kinase-1 (IRAK-1), as a critical modulator of macrophage migration. Macrophage migration induced by lipopolysaccharide (LPS) was significantly attenuated in IRAK-1−/− macrophages as compared to wild type (WT) macrophages. Mechanistically, this study demonstrated that IRAK-1 works downstream of IKKe and is required for LPS-induced expression of monocyte chemotactic protein-1 (MCP-1), a member of the chemokine family specific to macrophage migration, via C/EBPδ. The inhibition or knock-down of IKKe resulted in a significant decrease in the expression of C/EBPδ as well as MCP-1. Additionally, this study determined that the N-terminus of IRAK-1 forms a close complex with IKKe. The mutant IRAK-1-ΔN failed to associate with IKKe. These findings provide a novel facet regarding the molecular signaling process regulating macrophage migration.
INTRODUCTION

Macrophage migration is a highly integrated process which is regulated at multiple levels by diverse molecules including cellular receptors, intracellular signaling proteins, and cytoskeleton structural molecules (Raftopoulou and Hall, 2004; Ridley et al., 2003). The regulated expression of cytokines, chemokines, and chemokine receptors during immune cell activation and migration have been extensively studied in the past (Schilling et al., 2009; van der Meulen et al., 2009). However, relatively little information is available regarding the regulation of structural proteins by innate immunity signaling processes.

Chemokines belong to a large family of small chemoattractant cytokine proteins which regulate cell trafficking. Chemokines are subdivided into four families based on the number and spacing of the conserved cysteine residues in the N-terminus of the molecule and are named CXC, CC, CX3C, and C. MCP-1/CCL2 is one of the key members of the CC chemokine family and its main function is to regulate migration and infiltration of monocytes and macrophages (Deshmane et al., 2009; Linton and Fazio, 2003). MCP-1 can be produced by many different cell types including endothelial cells, fibroblasts, epithelial cells, smooth muscle cells, mesangial cells, astrocytes, monocytes and microglial cells, among which monocytes and macrophages are proved to be the main source (Yoshimura et al., 1989). MCP-1 is now the most widely studied chemokine and has been shown to be involved in many diseases such as multiple sclerosis (Sorensen et al., 2004), rheumatoid arthritis (Hayashida et al., 2001), and atherosclerosis (Boisvert, 2004).

IRAK-1 is a critical protein kinase involved in innate immunity signaling processes (Gan and Li, 2006; Ringwood and Li, 2008). IRAK-1 is known to not only be involved in the inducible
expression of pro-inflammatory mediators (Hu et al., 2002), but also in macrophage migration (Kramer et al., 2009). IRAK-1 deficient mice have decreased monocyte/macrophage infiltration to inflamed brain tissues in the EAE model (Deng et al., 2003). In addition, IRAK-4, another member of the IRAK family, is similar in that it is involved in neutrophil migration (Bouma et al., 2009) and LPS-induced local neutrophil influx (Koziczak-Holbro et al., 2009). However, the molecular mechanism underlying IRAK-1-mediated macrophage migration is not clearly understood. A recent study shows that LPS can induce MCP-1 production (Anand et al., 2009; Wang and Ohura, 2002), which offers that the Toll-like receptor 4 pathway may also play an important role in regulating MCP-1 expression.

In this report, we tested the hypothesis that IRAK-1 may be a close interacting partner with IKKe. Furthermore, we tested the functional relationships between IRAK-1 and transcription factor C/EBPδ, as well as its role in macrophage migration.
MATERIALS AND METHODS

Reagents

LPS (E. coli O111:B4) was obtained from Sigma. The antibodies against NFκB (ChIP), C/EBPδ, Lamin B, and GAPDH were purchased from Santa Cruz Biotechnology. The antibody against NFκB (western blot) was purchased from Cell Signaling Technology. The antibody against IRAK-1 was purchased from Millipore. IKKε inhibitor, 5-(5,6-Dimethoxybenzimidazol-1-yl)-3-(2-methanesulfonyl-benzyloxy)-thiophene-2-carbonitrile was from Calbiochem.

Mice, high-fat diet feeding, and culture of murine bone marrow-derived macrophage (BMDMs) and splenocytes

Wild-type C57BL/6 mice were obtained from the Charles River Laboratory. IRAK1−/− mice with C57BL/6 background were kindly provided by Dr. James Thomas from the University of Texas Southwestern Medical School. All mice were housed and bred at Derring Hall Animal Facility in compliance with approved Animal Care and Use Committee protocols at Virginia Polytechnic Institute and State University. Apoe−/− and Apoe−/−/IRAK-1−/− mice were fed with high-fat diet (Harlan Teklad 94059) for 3 month as previous described (Wang et al., 2008). BMDMs and splenocytes were harvested and cultured as we previously described (Su et al., 2007).

Trans-well cell migration assay

Trans-well migration assays were performed using 8-μm pore size trans-well inserts according to the manufacturer’s instructions (BD Biosciences). Briefly, BMDMs (4×10⁵) suspended in serum free DMEM medium were added to the top chambers of the trans-well inserts in a 24-well plate.
LPS was added to the lower chamber which contains DMEM/1% FBS. Cells were incubated for a proper amount of time, fixed, and then stained with crystal violet solution (Sigma). The numbers of migrated cells were counted under microscope and expressed as mean ± standard deviation.

**Isolation of cytoplasmic and nuclear extracts, and western blot**

Cell lysates, as well as the isolation of total, cytoplasmic, and nuclear extracts, were performed as described previously (Huang et al., 2004). Briefly, various cells (6×10^6/ml) were washed in phosphate-buffered saline (PBS) and subsequently lysed on ice in lysis buffer (10mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin). After centrifugation for 10 min at 6000 rpm, the supernatant cytoplasmic fractions were transferred and saved as cytoplasmic extracts. Pellets containing intact nuclei were lysed and solubilized with the high salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.4 M NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) for 30 min and yielded the nuclear extracts. Isolation of whole cell lysates was performed as described earlier(Su et al., 2007). Briefly, untreated or treated BMDMs were rinsed in PBS and then lysed on ice in 1x SDS lysis buffer (80 mM Tris-HCl, pH 6.8, 2% SDS, 50% glycerol) containing a protease inhibitor cocktail. Western blotting analysis of the protein samples were performed and immunoblots were developed by using the Amersham ECL Plus chemiluminescent detection system (GE Healthcare).

**Cell transfection and co-immunoprecipitation (co-IP)**

MAT4 (Hela cells stably transfected with TLR4 and MD2) cells were cultured in complete
DMEM medium supplemented with 10% FBS and antibiotics. Cells were transfected with the WT pFlag-IRAK-1 plasmid, the N-terminal deletion pFlag-IRAK-1ΔN plasmid, the C-terminal deletion pFlag-IRAK-1ΔC plasmids, or the pFlag-IRAK-1 (L167A/W168A) mutant plasmids. 24 h after the transfection, the cells were treated with 100ng/ml LPS for 15 min. Total cell lysates were prepared using lysis buffer (50mM HEPES, pH 7.6, 150mM NaCl, 0.5% NP-40, 1mM EDTA, 100 x protease inhibitors), and 800μg of cell lysate was used to perform immunoprecipitation with a specific antibody or an isotype control antibody from the same company (Santa Cruz). Co-immunoprecipitates were separated on SDS-PAGE and probed for the presence of various IRAK-1 forms using anti-Flag antibody.

**Real-time RT-PCR**

Total RNA was extracted from untreated or treated BMDMs using TRIZol (Invitrogen) according to the manufacturer’s protocol. Reverse transcription was carried out using the high-Capacity cDNA Reverse transcription kit (Applied Biosystems) and subsequent real-time RT-PCR analyses were performed using the SYBR green supermix on an IQ5 thermocycler (Bio-Rad). The relative levels of transcripts were calculated using the ΔΔCt method after normalizing with GAPDH as the internal control. The primer sets were obtained from IDT. The primer sequences are as follows: MCP-1(+), 5’-AGCCAACTCCTCAGTGAAG-3’, and MCP-1 (-), 5’-TGGAAAAGGTTAGTGGATG-3’; GAPDH (+), 5’-AACTTTGCGATTG GGAAGGCTCG-3’, and GAPDH (-), 5’-TGGAAAGAGGTGGGGAGTTGCTGT-3’.

**Transfection of small interfering RNAs (siRNAs)**
WT BMDM cells (2 × 10^6 cells) were plated in 6-well plates and transfected the following day using Lipofectamine 2000 (Invitrogen) with the indicated siRNA oligonucleotides (Santa Cruz Biotechnology). After 36 h post-transfection, the cells were treated with LPS (100 ng/ml) for the indicated time points, followed by RNA extraction and real-time RT-PCR (Maitra et al., 2009).

Chromatin Immunoprecipitation assay (ChIP)

WT and IRAK1^-/-BMDM cells were either untreated or treated with LPS (100 ng/ml) for 4h followed by cross-linking with 1% formaldehyde in complete media for 10 min with gentle rocking at room temperature. Cells were then washed twice with ice-cold PBS and treated with glycine solution for 5 min to stop the cross-linking reaction. Cells were then lysed in buffer containing SDS and protease inhibitor mixture. Samples were sonicated seven times with 30s pulses at 4°C followed by centrifugation to collect the sheared chromatin. The sheared chromatin was used to set up immunoprecipitation reactions with the indicated antibodies using the ChIP-IT Express kit (Active Motif) as per the manufacturer’s recommendations. The immunoprecipitated DNA fragments were analyzed by PCR using the primers spanning the binding sites of the specified transcription factors on the murine MCP-1 promoter as follows: C/EBPδ1(+), 5'-TCTGGTAACCACCAAGTGGAGAG-3' and C/EBPδ1(-), 5'-GCTTGGAAACACAGCCTAGCTTG-3'; C/EBPδ2(+), 5'-CCGTGTCACCTGTGTTACCTATGG-3' and C/EBPδ2(-), 5'-TTCCTGATTGGCGAGTTGTACCC-3'; NFκB(+), 5'-GATCTGAGAATGGGGAGTAACAGC-3' and NFκB(-), 5'-CCTGGAAAGCCATATARCGAAGTGGGGAGTAACAGC-3' and NFκB(-), 5'-CCTGGAAAGCCATATARCGAAGTGGGGAGTAACAGC-3'.

Statistical analyses

Statistical significance was determined using the unpaired 2-tailed Student’s t-test. P-values <
0.05 were considered statistically significant.
RESULTS

IRAK-1 contributes to MCP-1 protein production

Since the chemokine MCP-1 plays a key role in cell migration and LPS can induce the expression of MCP-1 (Anand et al., 2009), we then examined whether IRAK-1 participates in LPS-induced MCP-1 production. Using the multi-plex assay to measure the protein levels of MCP-1, we observed that overnight LPS treatment caused a significant production of MCP-1 in WT BMDM cell supernatant, but not in supernatant from IRAK-1−/− BMDM (Figure 1A).

Aside from the in vitro study, we examined the in vivo data. LPS was injected intraperitoneally into WT and IRAK-1−/− mice and the MCP-1 levels were measured in the collected blood plasma samples after 16h. As shown in Figure 1B, MCP-1 protein levels were higher in WT mice plasma than that in IRAK-1−/− mice after LPS treatment. In addition, it has been suggested that long-term high-fat feeding may chronically increase circulating levels of plasma LPS, which is defined as metabolic endotoxemia (Cani et al., 2007). Thus, we fed Apoe−/− and Apoe−/−/IRAK-1−/− mice with high-fat diet (HFD) and then measured the MCP-1 levels in blood plasma samples. As we expected, we detected decreased levels of MCP-1 in Apoe−/−/IRAK-1−/− mice blood compared to that in Apoe−/− mice (Figure 3C). Taken together we can conclude that the knock-out of IRAK-1 leads to a decrease in MCP-1 production.

IRAK-1 contributes to LPS-induced expression of MCP-1 mRNA

Since our study indicates that IRAK-1 deficiency ablates the induction of MCP-1 following both the in vitro and in vivo LPS treatments, we then tested the hypothesis that IRAK-1 may be required for the LPS-induced expression of MCP-1 mRNA. WT and IRAK-1−/− BMDM were
stimulated with LPS for 2h or 6h. Total RNAs were harvested and real-time RT-PCR was used to measure the levels of MCP-1 message. As shown in Figure 2A, the induction of MCP-1 by LPS was significantly lower in IRAK-1−/− BMDMs compared to WT BMDMs. In addition, total RNAs from splenocytes of high-fat feeding Apoe−/− and Apoe−/−/IRAK-1−/− mice were harvest and RT-PCR was performed. Interestingly, we found that the inductions of MCP-1 in splenocytes by high-fat feeding were decreased in Apoe−/−/IRAK-1−/− mice compared to Apoe−/− mice (Figure 2B). Therefore, IRAK-1 contributes to LPS-induced expression of MCP-1 mRNA.

**IRAK-1 mediates LPS-induced expression and activation and of C/EBPδ not NFκB**

After reviewing the promoter sequences of MCP-1, we found some potential putative C/EBPδ and NFκB binding sites within the proximal promoter of MCP-1 (Figure 3A), which is consistent with previous finding. We therefore examined the status of C/EBPδ in WT and IRAK-1−/− macrophages. LPS treatment led to a significant induction of C/EBPδ protein levels in whole cell lysates (Figure 3B) as well as nuclear lysates from WT cells (Figure 3C). Strikingly, LPS-mediated C/EBPδ induction was significantly impaired in IRAK1−/− cells. Thus, we examined the *in vivo* binding of C/EBPδ to the proximal promoter of MCP-1 in WT and IRAK1−/− BMDMs using chromatin ChIP analysis. As shown in Figure 3D and 3E, there was no basal interaction of C/EBPδ with the MCP-1 promoter. LPS treatment led to a significant recruitment of C/EBPδ to the endogenous MCP-1 promoter in WT BMDMs. In contrast, the binding of C/EBPδ to the MCP-1 promoter in response to LPS treatment in IRAK-1−/− BMDMs was dramatically reduced (Figure 3D and 3E). In contrast, it became evident that there was almost the same amount of binding between NFκB to MCP-1 promoters in WT and IRAK1−/− BMDMs (Figure 3F). Therefore, IRAK-1 mediates LPS-induced C/EBPδ but not NFκB
expression and activation.

**IRAK-1 interacts with IKKε**

In order to examine the molecular mechanism for the IRAK-1-mediated expression of MCP-1, we examined whether IRAK-1 and IKKε form a close complex following LPS treatment. IKKε-associated proteins were immunoprecipitated with anti-IKKε antibody in WT BMDMs. As shown in Figure 4A, there was obvious co-immunoprecipitation of IRAK-1 and IKKε with and without LPS treatment.

We further studied the protein domain(s) required for the interaction between IRAK-1 and IKKε. Flag-tagged WT and mutant IRAK-1 plasmids (Figure 4B) were transiently transfected into MAT-4 cells. Co-immunoprecipitation analyses were performed using the anti-IKKε antibody as described in the Materials and Methods section. Co-immunoprecipitates were resolved on SDS-PAGE, gels and blotted with either the anti-Flag antibody (detecting various IRAK-1 forms) or the anti-IKKε antibody. As shown in Figure 4C, LPS treatment led to co-immunoprecipitation of IKKε with the full length Flag-IRAK-1, Flag-IRAK-1 mutation (L167A/W168A) and the Flag-IRAK-1ΔC. Strikingly, the Flag-IRAK-1ΔN failed to co-immunoprecipitate with IKKε. This data indicates that the N-terminal region of IRAK-1 molecule is involved in its interaction with IKKε.

**IRAK-1 helps to IKKε-mediated expression of C/EBPδ and MCP-1.**

Therefore, we planned to further confirm IRAK-1 function on downstream C/EBPδ and MCP-1 expression. IKKε specific inhibitors as well as IKKε siRNA were used to inhibit or knock out
IKKε. BMDM were treated with inhibitor or siRNA transfection first. LPS was then later added and real-time RT-PCR was used to measure the mRNA levels of MCP-1. Strikingly, we observed that the impairment of IKKε resulted in reduction of MCP-1 expression in WT cells but not in IRAK1−/− cells, which suggests that IRAK-1 is upstream of IKKε (Figure 5A, 5B). IKKε mRNA levels confirmed the siRNA technique (Figure 5C). In addition, LPS-induced C/EBPδ expression was blocked by an IKKε inhibitor in WT cells but not in IRAK-1−/− cells (Figure 5D). This confirms that IRAK-1 functions upstream of IKKε. In conclusion, we have determined that LPS-induced CEBP-δ as well as MCP-1 expression is IRAK-1 dependent.

**IRAK-1 is involved in LPS-induced macrophage migration**

To determine the direct role of IRAK-1 in LPS-induced BMDM migration, we performed trans-well migration assays using WT and IRAK-1−/− BMDMs. Following LPS treatment, cells attached to the membrane were stained with crystal violet dye. As shown in Figure 6, LPS treatment significantly increased induction of cell migration in WT BMDM. In contrast, there was a 30% reduction in cell migration with IRAK-1−/− BMDM.
DISCUSSION

In this study, we have defined a novel role for IRAK-1, a key TLR4 signaling component, in modulating LPS-induced macrophage migration. IRAK-1 fulfills this function by inducing the expression of MCP-1 both \textit{in vitro} and \textit{in vivo} and interacts with IKK\textepsilon to regulate expression and activation of C/EBP\textgreek{d}. Collectively, IRAK-1\textsuperscript{-/-} BMDM cells exhibit reduced migration ability following LPS challenge (Figure 7).

This data is consistent with several recent studies showing the involvement of C/EBPs in LPS-mediated production of MCP-1 in macrophages (Hu et al., 2000; Spooner et al., 2007a; Spooner et al., 2007b). Since IRAK-1 acts upstream of IKK\textepsilon within the LPS signaling pathway, it is conceivable that IRAK-1 participates in the LPS signaling process controlling the expression of MCP-1. This study reveals the contribution of IRAK-1 in LPS-induced macrophage migration and in the regulation of MCP-1. In particular, our data indicates that IRAK-1 facilitates the generation of MCP-1 in macrophages by modulating the transcription factor C/EBP\textgreek{d} but not NF\textkappa B, as was traditionally thought (Figure 3). Our current study confirms and expands upon previous findings, and provides the first biochemical evidence indicating that IRAK-1 physically associates with IKK\textepsilon upon LPS challenge and is upstream of MCP-1 expression. Furthermore, this data verifies that the N-terminal but not the LWPPPP motif of IRAK-1 is required for its interaction with IKK\textepsilon (Figure 4). However, its functional implication is still poorly understood.

The expression of MCP-1 is known to be highly inducible following LPS treatment. Therefore, both the activation of C/EBP\textgreek{d} via IKK\textepsilon and the induced expression of MCP-1 contribute to the elevated levels of macrophage migration, especially in WT cells. Our study demonstrates that
IRAK-1 is critically involved in the inducible expression of MCP-1, potentially via C/EBPδ. This finding also expands the repertoire of IRAK-1 downstream signaling components and reveals C/EBPδ as a novel target of IRAK-1. In addition, we proved that IRAK-1 indirectly contributes to the activation of C/EBPδ, which is consistent with previous findings showing that IKKε is required for post-transcriptional regulation of C/EBPδ. Other research has also shown that LPS can induce IKK ε protein expression (Kravchenko et al., 2003). Moreover, it has been identified that the putative binding sites for C/EBPδ within the proximal promoter of MCP-1 might regulate its expression. Interestingly, LPS-mediated C/EBPδ induction is significantly impaired in IRAK1−/− cells as compared to WT cells. Further studies are required to clarify the underlying mechanism.

Historically, NFκB is the most extensively studied transcription factor downstream of TLR4 (Deshpande et al., 1997; Saban et al., 2002; Zuckerman et al., 1991). However, it has been increasingly recognized that NFκB alone is usually not sufficient for effective transcription of selected target genes. Intriguingly, our data shows almost equal binding of NFκB to the MCP-1 promoter in WT and IRAK-1−/− cells, which demonstrates that IRAK-1 does not contribute to LPS-induced MCP-1 expression via NFκB.

Collectively, this study reveals the potential mechanism underlying the beneficial effects observed in animal models with IRAK-1 gene deletion. Previous studies have indicated that the IRAK-1-deficiency protects mice from various inflammatory diseases including EAE (Deng et al., 2003), endotoxemia (Swantek et al., 2000), atherosclerosis (Thomas et al., 2003), diabetes (Lakoski et al., 2007), and systemic lupus erythematosus (Jacob et al., 2009). Our current study further reveals that IRAK-1-deficient cells undergo significantly less migration coupled with a
decrease in MCP-1 expression following LPS treatment. This data provides compelling evidence indicating that IRAK-1 is not only a key mediator for LPS-induced production of pro-inflammatory cytokines, but it can also regulate macrophage migration. Taken together, we postulate that IRAK-1 can serve as a viable target for future intervention of inflammatory diseases.

ACKNOWLEDGEMENTS

This work is supported by research grants from the National Institute of Health. We thank Samantha Chang for proof reading.
FIGURE LEGENDS

Figure 1: IRAK-1 contributes to MCP-1 protein production. A. WT and IRAK-1\(^{-/-}\) BMDM were treated with LPS (100ng/ml). After 16 h, cell supernatants were collected and subjected to multi-plex assay. B. Ten mice of WT or IRAK-1\(^{-/-}\) groups were injected with LPS (25mg/kg body weight) or PBS (vehicle) intraperitoneally. After 16 h, plasma samples were collected from WT and IRAK1\(^{-/-}\) mice and the MCP-1 concentration was measured using multi-plex assay. C. Ten mice of Apoe\(^{-/-}\) or Apoe\(^{-/-}/\)IRAK-1\(^{-/-}\) groups were fed with high-fat diet as described in materials and methods for 3 month and the MCP-1 concentrations in plasma were measured using multi-plex assay. Each data point represents the mean S.D. of at least three independent experiments. *\(P<0.05\), compared with control.

Figure 2: The effect of LPS on MCP-1 mRNA expression in WT and IRAK1\(^{-/-}\) BMDM cells. A. BMDMs were stimulated with LPS (100 ng/ml) for 2 h or 6 h. After stimulation, total RNA was isolated using TRIZol reagent and then reverse transcribed. The mRNA levels of MCP-1 were then analyzed using real-time RT-PCR. B. Splenocytes were harvest after high-fat feeding, total RNA was isolated and real-time RT-PCR was then performed. Each data point represents the mean S.D. of at least three independent experiments. *\(P<0.05\), compared with control.

Figure 3: IRAK-1 mediates LPS-induced activation of C/EBP\(\delta\) not NF\(\kappa\)B. A. Putative binding sites of transcription factors within the proximal promoter of murine MCP-1, 1+ denotes the transcription start site. B. WT and IRAK1\(^{-/-}\) BMDM cells were treated with 100 ng/ml LPS for the indicated time points. Equal amounts of total cell lysates were resolved on SDS-PAGE gels and blotted with antibodies specific for C/EBP\(\delta\) and NF\(\kappa\)B. Data are representative of three independent experiments. The blots were stripped and then re-probed with GAPDH as the
loading control. C. Nuclear extraction samples were revolved on SDS-PAGE gels and blotted with antibodies specific for C/EBPδ and NFκB. The blots were stripped and then re-probed with Lamin B as the loading control. D, E, F. Decreased binding of C/EBP δ, but not NFκB to the proximal promoter of murine MCP-1 in IRAK1−/− BMDMs compared to WT BMDMs. The BMDMs were either un-stimulated or induced with LPS for 4 h and subjected to chromatin ChIP assay using the indicated antibodies and primers specific to the proximal promoter of murine MCP-1. C/EBPδ(1/2) refer to the different primer sets for two potential C/EBPδ binding sites. Data are representative of three independent experiments.

**Figure 4: IRAK-1 interacts with IKKε.** A. WT murine BMDM cells were either un-stimulated or treated with LPS (100ng/ml) for 15 min. Equal amounts of total cell lysates were used to perform co-IP analyses using an anti-IRAK-1 antibody. Co-immunoprecipitated protein complexes were resolved on a SDS-PAGE gel and blotted with an anti-IKKε antibody (top panel). An isotype control antibody from the same company was used to perform a similar immunoprecipitation study, which gave a few signals near the IKKε region. The levels of IRAK-1 in the IP complex are shown in the bottom panel. B. A diagrammatic illustration of various FLAG-tagged IRAK-1 full-length and deletion constructs used in the transfection studies. C. MAT4 cells were transiently transfected with pFLAG-IRAK-1, pFLAG-IRAK-1 Δ N, pFLAG-IRAK-1ΔC, or pFLAG-IRAK-1 (L167A/W168A) mutant. Equal amounts of lysates were harvested from the transfected cells and used to perform IP analyses using an anti-IKKε antibody. Co-immunoprecipitated protein complexes were resolved on SDS-PAGE gels and blotted with an anti-FLAG antibody (top panel). An isotype control IgG was used to perform a similar immunoprecipitation study and did not give a nonspecific signal near the region of
interest. The expression levels of IKKε in the IP complex and various FLAG-IRAK-1 mutants within the cell lysates are indicated in the bottom panels and the upper left panels. The data represent three independent experiments.

**Figure 5:** IRAK-1 is required for IKKε-mediated expression of C/EBPδ and MCP-1. 

A. WT and IRAK1−/− BMDMs were stimulated with 200nM of IKKε inhibitor (IKKεi) for 1 h, and then stimulated with LPS (100ng/ml) for 4 h. MCP-1 mRNA levels were measured by real-time RT-PCR. 

B. WT BMDMs were used to perform IKKε knockdown by siRNA first. After 36 h, RNA was harvested and MCP-1 mRNA levels were measured. 

C. The same mRNA samples were used to test IKKε mRNA levels. 

D. WT and IRAK1−/− BMDMs were stimulated with IKKε inhibitor for 1 h followed by LPS treatment, and then whole cell lysates were isolated. The lysates were resolved on SDS-PAGE gels and analyzed using an anti-C/EBPδ antibody as indicated. The same blots were re-probed with GAPDH as the loading control.

**Figure 6:** Depletion of IRAK-1 impairs cell migration. 

A. BMDMs from WT and IRAK1−/− mice were loaded onto the top wells of the trans-well chamber with or without LPS (100ng/ml) added to the bottom chamber. After incubation in a humidified 5% CO2 incubator for 16 h, the cells on the lower surface of the filter were visualized by microscopy after staining with crystal violet solution. The transmigrated cells were photographed in triplicate wells at 20× magnification. 

B. Cell migration was determined as the number of cells that had migrated across the filter in five photographed areas (A). Results shown are mean±SEM. *P < 0.05. These findings are similar to those obtained in two other independent experiments.

**Figure 7:** A schematic diagram illustrating the contribution of IRAK-1 to LPS-induced
MCP-1 expression and cell migration. LPS can induce MCP-1 production via transcription factor NFκB or C/EBPδ. IRAK-1 contributes to MCP-1 production only by C/EBPδ instead of NFκB.
Figure 1

A

WT
IRAK-1-/-

MCP-1 (pg/ml)

WT
IRAK-1-/-

Control LPS

B

MCP-1 (pg/ml)

WT
IRAK-1-/-

Control LPS

C

Apoe-/-
Apoe-/-/IRAK-1-/-

MCP-1 (pg/ml)

Apoe-/-
Apoe-/-/IRAK-1-/-

*
Figure 2

A

**Relative levels of MCP-1 mRNA in BMDMs**

- **WT**
- **IRAK-1-/—

B

**Relative levels of MCP-1 mRNA in Splenocytes**

- **Apoe-/—**
- **Apoe-/—/IRAK-1-/—**
Figure 3

A. Schematic representation of the MCP-1 promoter region (-1331 to +2) showing the positions of the binding sites for C/EBPδ and NFκB(p65).

B. Western blot analysis of C/EBPδ, IκBα, and GAPDH in WT and IRAK-1-/- cells stimulated with LPS for 0, 2, and 4 hours.

C. Western blot analysis of C/EBPδ, p65, and Lamin B in WT and IRAK-1-/- cells stimulated with LPS for 0, 2, and 4 hours.

D. ChIP analysis of C/EBPδ(1) in WT and IRAK-1-/- cells stimulated with LPS for 0, 2, and 4 hours.

E. ChIP analysis of C/EBPδ(2) in WT and IRAK-1-/- cells stimulated with LPS for 0, 2, and 4 hours.

F. ChIP analysis of NFκB p65 in WT and IRAK-1-/- cells stimulated with LPS for 0, 2, and 4 hours.
Figure 4

A

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C

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| Flag IRAK-1ΔC   |         |     |
| IgGh            |         |     |
| IKKε            |         |     |
| IgGh            |         |     |
Figure 5

A

Relative expression of MCP-1 mRNA

WT IRAK-1-/

Control

LPS

IKKε+LPS

B

Relative expression of MCP-1 mRNA

WT IRAK-1-/

Control

IKKε siRNA

Control+LPS

IKKε siRNA+LPS

C

Relative expression of IKKε mRNA

WT IRAK-1-/

Control

IKKε siRNA

D

WT IRAK-1-/

IKKcili

C/EBPδ

GAPDH
Figure 6

A

WT Control

IRAK-1/- Control

WT LPS

IRAK-1/- LPS

B

WT

IRAK-1/-

Migrated Cells

0

400

800

1200

1600

Control

LPS

*
Figure 7

Transcription factors

Downstream genes:
Cell migration regulator

IRAK1 knockout

Cell migration

C/EBP-δ

MCP-1

IKKε

IKKα/β

NFκB

IRAK4

IRAK2
REFERENCE


stromal cells from rheumatoid arthritis patients attract monocytes by producing MCP-1 and IL-8. Arthritis research 3, 118-126.


IRAK-1 interacts with the small GTPase Rac1, and contributes to LPS-induced activation of Rac1

Murine macrophages express two of the three Rac isoforms, Rac1 and Rac2. Since the small GTPase Rac1 plays a key role in the rapid activation of LPS-induced NADPH oxidase, we then studied whether IRAK-1 participates in LPS-induced Rac1 activation and the molecular mechanism for the IRAK-1 mediated activation of Rac1. We examined whether IRAK-1 and Rac1 may form a close complex following LPS treatment. WT BMDMs were stimulated with LPS for 5 minutes, and IRAK-1 associated proteins were immunoprecipitated with the anti-IRAK-1 antibody. As shown in Figure 1A, LPS treatment induced dramatic co-immunoprecipitation of IRAK-1 and Rac1.

We further studied the protein domain(s) required for the interaction between IRAK1- and Rac1. Flag-tagged WT and mutant IRAK-1 plasmids were transiently transfected into MAT4 cells (Figure 1B). Transfected cells were treated with LPS for 5 minutes. Co-IP analyses were performed using the anti-Flag antibody as described in the Materials and Methods. Co-immunoprecipitates were resolved on SDS-PAGE, and blotted with either the anti-Flag antibody (detecting various IRAK-1 forms) or the anti-Rac1 antibody. As shown in Figure 1C, LPS treatment led to co-immunoprecipitation of Rac1 with the full length Flag-IRAK-1 and the Flag-IRAK-1ΔC. Strikingly, we observed that either Flag-IRAK-1ΔN or Flag-IRAK-1 (PPXXAA) failed to co-immunoprecipitate with Rac1. Our data indicate that the N-terminal region of IRAK-1 molecule is involved in its interaction with Rac1. In particular, the proline rich motif within the N-terminal region is responsible for the close association with Rac1.
Rac1 has known to play an essential role in cell migration at multiple steps during migration cycle. Since IRAK-1 can interact with Rac1 and contribute to Rac1 activation, it is highly likely that IRAK-1 is also involved in cell migration by regulating Rac1 activity. However, further experiments are needed to clarify this relationship.
Figure 1 IRAK-1 interacts with Rac1. A. WT murine BMDM cells were either untreated or treated with 100 ng/ml LPS for 5 min. Equal amounts of total cell lysates were used to perform immunoprecipitation (IP) analyses using an anti-IRAK-1 antibody. Co-immunoprecipitated protein complexes were resolved on SDS-PAGE gels and blotted with an anti-Rac1 antibody (top panel). An isotype control antibody from the same company was used to perform a similar immunoprecipitation study, which gave no signal near the Rac1 region (data not shown). The levels of IRAK-1 in the cell lysates are shown in the bottom panel. IB, immunoblot. B. A diagrammatic illustration of various FLAG-tagged IRAK-1 full-length and deletion constructs used in the transfection studies. C. MAT4 cells were transiently transfected with pFLAG-IRAK-1, pFLAG-IRAK-1ΔN, pFLAG-IRAK-1ΔC, or pFLAG-IRAK-1(L167A/W168A) mutant. Equal amounts of lysates were harvested from the transfected cells and used to perform immunoprecipitation analyses using an anti-Rac1 antibody. Co-immunoprecipitated protein complexes were resolved on SDS-PAGE and blotted with an anti-FLAG antibody (top panel). An isotype control rabbit IgG was used to perform a similar immunoprecipitation study and did not give a nonspecific signal near the region of interest. The expression levels of Rac1 and various FLAG-IRAK-1 mutants within the cell lysates are indicated in the bottom panels. The data represent three independent experiments.

(This work was published in Maitra U, Singh N, Gan L, Ringwood L and L. Li, 2009, J Biol Chem, 284(51): 35403-41)
CHAPTER 7: IMPLICATIONS AND FUTURE DIRECTIONS

The central objective of this project was to elucidate the role of IRAK-1 in macrophage migration. *In vitro* trans-well cell migration assays in BMDM cell cultures were undertaken to assess the effect of IRAK-1 on cell migration signaling pathway. A secondary objective was to discern the molecular mechanism in which IRAK-1 is involved in LPS-induced migration of murine macrophages.

During PMA stimulation, we observed a reduction in macrophage migration capability in IRAK-1-deficient cells. Interestingly, IRAK-1 interacts with both PKCε and its substrate VASP to regulate downstream cell migration. These results disclose a novel pathway which suggests an indirect function of PKCε on VASP phosphorylation through IRAK-1. However, this is not the only pathway utilized, since IRAK-1 deficiency does not completely block VASP phosphorylation or microphage migration. This finding is consistent with previous studies showing the direct role of PKCε on VASP phosphorylation.

As for LPS challenge, IRAK-1-deficient cells again showed a reduced cell migration ability compared to WT cells. This was closely related to the association between IRAK-1 and IKKe. MCP-1 expression induced by LPS was also elevated in WT cells compared to IRAK-1-deficient cells. It was hypothesized that IRAK-1 serves as a positive regulator of inflammation as well as cell migration, which suggests that IRAK1 may prove to be an important future therapeutic target.
As for Rac1, further studies are needed to completely clarify its relationship and interaction with IRAK-1. Our preliminary data shows that the IRAK-1 LWPPPP motif within the N-terminal directly binds to Rac1. Interestingly, this is also the binding site for VASP to IRAK-1. These findings further confirm that IRAK-1 function is important in macrophage migration.

Taken altogether, cell migration is regulated by multiple pathways and there are likely multiple points of crosstalk between these different pathways. IRAK-1 is not only a signaling molecule involved in the IL-1/TLR pathway, but is also involved in the regulation of actin cytoskeletons. Our research opens a brand new world in which innate immune pathways are closely linked to cell motility, and points to the possibilities for anti-inflammatory therapies through the regulation of IRAK-1 function. Further studies on this topic will be highly essential in the future.
CHAPTER 8 APPENDICES

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