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Innate immune responses to B. burgdorferi mediated by JNK1 and the cochaperone, methylation controlled DNAJ (MCJ)

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INNATE IMMUNE RESPONSES TO *B. BURGDORFERI* MEDIATED BY JNK1 AND THE COCHAPERONE, METHYLATION CONTROLLED DNAJ (MCJ)

A Dissertation Presented

by

HOOMAN IZADI

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DEDICATED TO

This dissertation and degree is dedicated to my late father Dr. Manoutchehr Izadi who always believed in me and provide every opportunity and resources that I have needed to succeed in my earlier education and life, which are the basis for my current success and professional development. I imagine and know how proud and happy you would be if you were around in this planet today.

I would also like to dedicate this work to my mother Vahideh Izadi and my brother Ramin Izadi for their unconditional love, encouragement and support. I love you so much with everything that I have and striving to have.

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ABSTRACT

INNATE IMMUNERESPONSES TO *B. BURGDORFERI* MEDIATED BY JNK1 AND THE COCHAPERONE, METHYLATION CONTROLLED DNAJ (MCJ)

FEBRUARY 2011

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The infections agent of Lyme disease, *Borrelia Burgdorferi* is a complex microorganism with a highly diverse genome. One of the most remarkable aspects of the *B. burgdorferi* genome is the large number of sequences encoding predicted or known lipoproteins, including outer-surface proteins. The *B. burgdorferi* genome encodes no recognizable toxins. Instead, this extracellular pathogen causes pathology by migration through tissues, adhesion to host cells, and evasion of immune clearance. Inflammation elicited by infection with *B. burgdorferi* depends on the ability of the spirochete to survive in the mammalian host, as well as the immune response that arises upon the interaction of the bacterium with phagocytic, T and other cell types. Innate immune responses are critical in recognition and clearance of pathogens, and also play an important role in the outcome of adaptive immune responses.

The regulation of innate immune responses to pathogens occurs through the interaction of Toll-like receptors (TLRs) with pathogen-associated molecular patterns

(PAMPs) and the activation of several signaling pathways whose contribution to the overall innate immune response to pathogens is poorly understood. In this study we demonstrate a mechanism of control of murine macrophage responses mediated by TLR1/2 heterodimers through c-Jun N-terminal kinase 1 (JNK1) activity. JNK also controls tumor necrosis factor production and TLR-mediated macrophage responses to *B. burgdorferi*. We also show that the proximal promoter region of the human *tlr1* gene contains an AP-1 binding site that is subjected to regulation by the kinase and binds two complexes that involve the JNK substrates c-Jun, JunD, and ATF-2. These results demonstrate that JNK1 regulates the response to TLR1/2 ligands and suggest a positive feedback loop that may serve to increase the innate immune response to the spirochete.

MCJ is a newly identified member of the DnaJ protein family of cochaperones that contains unique features different than the normally described DnaJ proteins. However, there is little known about its function and the role it plays in different cells and systems. It has been previously shown that MCJ is required for the repression of the ABCB1 drug transporter expression in breast cancer cells, and that this repression is mediated through the control of c-Jun protein stability. We were therefore interested in determining the role that MCJ plays in macrophages in response to *B. burgdorferi* antigens. We now provide evidence that MCJ controls inflammatory responses of macrophages through the regulation of c-Jun protein stability, and the expression and release of the inflammatory cytokine TNF, through the regulation of the expression of TNF converting enzyme (TACE) inhibitor tissue inhibitor of metalloproteinase 3 (TIMP-3).

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CHAPTER I

INTRODUCTION

Lyme disease was first discovered in 1975 as a tick-borne infection affecting a cluster of children in Lyme, Connecticut. In 1982, Willy Burgdorfer and co-workers indentified the agent of Lyme disease as a new spirochetal, extracellular bacterium (1). This spirochete was named *Borrelia burgdorferi*. Since then, at least three species of the genus *Borrelia* have been shown to be agents of Lyme disease. These include *B. burgdorferi sensu stricto,* B. *afzelii* and B. *garinii,* which as a group are referred to as *B. burgdorferi sensu lato* (2, 3). At least twelve other genospecies of *B. burgdorferi* that are minimally pathogenic or nonpathogenic have been identified, geographically spanning the United States and Eurasia (4, 5, 6). In the United States *B. burgdorferi* is the sole agent of Lyme disease; however *B. afzelii* and *B. garinii* cause the disease in areas of Europe and Asia (3, 7). In the United States, *B. burgdorferi* is transmitted by the hard-bodied ticks, *Ixodes scapularis* and *I. pacificus*. Lyme disease is the most reported arthropod-borne disease in United States.

Biology of *Borrelia* and Lyme disease

B. burgdorferi is a highly prevalent vector-transmitted spirochete. Spirochetes are a diverse and unique group of bacteria that can inhabit different environments like soil, arthropods and humans. Many spirochetal species are able to cause disease in mammals such as *Treponema pallidum*, *Borrelia burgdorferi* and *Leptospira spp* (8). Lyme disease is a zoonotic, vector-borne disease that is transmitted by *Ixodes* ticks. *Ixodes* ticks are

obligate blood-feeding ectoparasitic acari that belong to the large family *Ixodidae* (hard ticks). A diverse array of infectious agents of medical and veterinary importance are transmitted by *Ixodid* ticks (9). In particular, *I. scapularis* ticks act as vectors for several pathogens including *B. burgdorferi*, *Anaplasma phagocytophilum*, and *Babesia microti* (1, 10) and are second only to mosquitoes worldwide as vectors of human pathogens.

The life cycle of *I. scapularis*, commonly known as the black-legged tick, consists of three developmental stages: larva, nymph, and adult (11). It takes about two years for the life cycle of the black-legged tick to be completed. Adult female ticks lay eggs on the ground in early spring and by summer the eggs are hatched into larvae. Larval ticks feed on small animals such as mice, other rodents, deer, and birds and molt into nymphs. Nymphs feed on small animal and rodents as well as humans in the late spring and summer, and mature into adults. Adult ticks feed and mate on large mammals (especially deer). The adult females then drop from these animals and lay eggs in spring to complete the two-year cycle.

Humans and deer are not involved in the maintenance of *B. burgdorferi*, and thus are not part of the enzootic life cycle of the spirochete. Instead, both large mammals are incidental hosts, which acquire the infection from infected ticks questing for a blood meal. *B. burgdorferi* infects a wide range of vertebrate animals including small mammals, and birds (12, 13). Ticks most frequently acquire spirochetes from infected rodents during their larval feeding (13, 14). After molting to the nymphal stage, infected ticks feed on a broad range of animals including rodents, which become a new reservoir, perpetuating the cycle (15). The spirochetes are rarely, if ever, transmitted transovarially (16), making the nymphal stage the most significant for human transmission, which is

also aided by their small size that makes them difficult to detect and, therefore, more likely to feed long enough to transmit the spirochete (16, 17). *B. Burgdorferi* is most commonly transmitted to humans during late spring or early summer, which coincides with the nymphal feeding period. The tick must be attached for at least 24 to 48 hours in order to transmit the bacteria to humans (18). *B. burgdorferi* is well adapted to survive in two different environments, the tick and the mammalian host. *B. burgdorferi* must persist in the tick midgut for an extended period, post feeding, without nutrients. Upon tick feeding, *B. burgdorferi* adjusts protein expression to enable a successful infection of the vertebrate host. A well characterized paradigm in protein expression shift is that of OspA and OspC. OspA is expressed at high levels in the midgut of the unfed tick, but its expression is down regulated upon tick feeding, whereas OspC is up regulated (19).

During pathogenesis of Lyme disease, *B. burgdorferi* produces a number of products that allow it to colonize and persist in its natural mammalian and tick hosts. Although the functions of only a few *B. burgdorferi* products have been clearly defined, some such as (OspC) are required for the bacteria to survive the initial attack of the mammalian innate immune system, whereas others (such as VlsE) contribute to resisting the subsequent acquired immune response (20). These bacterial products allow it to replicate and survive. Although they are not true virulence factors, they seem to be adequate for the bacterium to cause disease in a susceptible host. In support of this idea, the genome sequence of B31, the type strain of *B. burgdorferi sensu stricto*, revealed that the bacterium lacks factors common to many bacterial pathogens, such as lipopolysaccharide, toxins, and specialized secretion systems. The bacterium does however; contain immunogens such as lipoproteins on its surface (19, 21).

A hallmark of infection with *B. burgdorferi* is the appearance of a skin rash known as *erythema migrans*, which is a sign of early infection and the most reported symptom associated with the disease (22). Other symptoms that may occur at this time include fever, headaches, malaise and myalgias. If untreated, the spirochete can disseminate through the blood (stage II, Lyme disease) and cause symptoms that include meningitis, conduction system abnormalities and acute arthritis. These symptoms appear in 60% of untreated individuals. Some untreated individuals may progress to stage III Lyme, also known as late persistent disease, a prolonged infection of unclear etiology with symptoms that can include chronic arthritis and neuroborreliosis (22, 23).

Overview of the immune response to *B. burgdorferi*

Vertebrates are constantly threatened by the invasion of multiple microorganisms and have evolved immune defense systems to detect and eliminate pathogens from the body. Mammals have two branches in their immune system: innate and adaptive immunity (24). The innate immune responses are the first line of defense against invading pathogens and include phagocytic cells such as macrophages and neutrophils. The recognition of *B. burgdorferi* by innate immune cells depends on the rapid detection of the microorganism, mediated by germline-encoded pattern recognition receptors (PRRs) that are critical to discriminate between host factors from infectious agents. These interactions are also important for determining the extent and quality of adaptive immune responses. Toll-like receptors (TLRs) are PRRs that detect foreign pathogens (25, 26). Following the recognition of *B. burgdorferi*, phagocytic cells are recruited to the site of infection, mediated by the initial production of cytokines and chemokines from cells at

site of the infection, which also initiates the up regulation of cell adhesion molecules in endothelial cells (27, 28).

Upon presentation of *B. burgdorferi* antigens by phagocytic and other antigen presenting cells, CD4+ T cells become differentiated effector cells. IFNγ-producing Th1 cells are regulators of cell-mediated inflammatory reactions, which are characterized by macrophage activation and production of opsonizing IgG antibodies. Infection with *B. burgdorferi* results in the production of IL-12 that drives differentiation of $CD4^+$ T cells toward a Th1 phenotype (29). Functional T cells are not required for resolution of infection with *B. burgdorferi*; however they contribute to the pathology associated with infection (30, 31). Moreover, during Lyme carditis, which is a *B. burgdorferi*-induced inflammatory phenomenon that involves predominantly macrophages, the activation of iNKT cells and aTh1 response are required for the clearance of *B. burgdorferi* and the resolution of disease (25, 32). In spite of intense efforts to determine the mechanisms that contribute to the ability of *B. burgdorferi* to cause persistent infection, even in the presence of strong immune responses, no clear mechanism has been unveiled to date (25).

Innate immunity and *B. burgdorferi*

A critical part of an effective host defense is the detection of microbial products by innate immune cells (33). Innate immune responses are mediated by several cell types, including macrophages (24). Originally, innate immune responses were thought to be nonspecific; however it is now known that they have some specificity and are able to differentiate between self and a variety of invading organisms. Innate immune cells can

recognize pathogens by a variety of PRRs (24, 34). There are several classes of PRRs such as toll like receptors (TLRs), Retinoic acid inducible gene (RIG) like receptors (RLRs) and NOD like receptor (NLRs).

These receptors are essential components of innate immune cell responses to pathogens. They recognize Pathogen-associated molecular patterns (PAMPs). PAMPs are molecules associated with groups of pathogens that can be referred to as small molecular motifs conserved within a class of microbes. Recognition of PAMPs initiates a cascade of responses that lead to the up regulation of cytokines (TNF, IL-12, IL-18, and IL-1β among others), chemokines (IL-8, MCP-1, KC) and adhesion molecules (E-selectin, VCAM-1 and ICAM-1) (24, 34, 35). These events result in the activation of phagocytic cells and the eventual development of adaptive immune responses (27, 36). One of the most studied PAMPs of *B. burgdorferi* recognized by innate immune cells is triacylated outer-surface lipoproteins. *B. burgdorferi* lipoproteins interact with the complex formed by TLR1 and TLR2 leading to the activation of several signaling cascades that result in the production of pro-inflammatory cytokines such as TNF and IL-12. Detection of *B. burgdorferi* lipoproteins is important for defining the *in vivo* responses to *B. burgdorferi* as demonstrated by the increased bacterial burdens in TLR1- or TLR2-defficent mice (35, 37, 38).

The Toll like Receptors (TLRs) and their signaling

TLRs are members of a larger super family of receptors (to which IL-1R also belongs) that are involved in recognition of microbial conserved structures. TLRs are

among the most conserved signaling components that are widely expressed on antigen presenting cells, especially macrophages and dendritic cells (39-41).

The TLR family is highly conserved evolutionarily from worms to mammals, and was found as essential for the development in *Drosophila* (24). Twelve members of the TLR family have been discovered in mammals. Toll like receptors are type I integral membrane glycoproteins and have a trimodular structure. The N-terminal domain, which is the extracellular portion, contains approximately 16-28 leucine rich repeats (LRRs), each LRR with 20-30 amino acids with conserved motifs (LxxLxxN). The C-terminal domain is the intracellular part of TLRs, known as Toll/IL-1 receptor (TIR) domain, which shows homology with the IL-1 receptor. Homology in the TIR domain is confined to three conserved boxes that contain amino acids crucial for signaling (24, 36, 42, 43). This part of TLR is required for the interaction and recruitment of many other adaptor molecules (44). Recently, the crystal structure of several TLRs with their ligands have been assembled, showing that they form heterodimer complexes such as TLR1-TLR2 or homodimers like TLR3-TLR3 after interaction with their specific ligand. These dimers assume a horseshoe-like structure that is required for ligand binding and the initiation of downstream signaling pathways (45, 46, 47).

Based on the primary sequences of TLRs, they can be divided in different subfamilies each recognizing specific PAMPs derived from various pathogenic bacteria, fungi and parasitic-protozoa. TLRs are expressed in distinct compartments of the cell. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on the cell surface whereas TLR3, TLR7, TLR8 and TLR9 are expressed in intracellular vesicles such as endosomes and the ER. TLRs can sense various components of the bacterial cell wall, such as LPS

from gram-negative bacteria by TLR4; diacylated or triacylated lipopeptides from bacteria recognized by TLR2/TLR6 and TLR1/2, respectively; flagellin by TLR5; and unmethylated CpG DNA by TLR9 (44).

Four adapter molecules are known to be involved in signaling mediated by TLRs: Myeloid differentiation 88 (MyD88), TIRAP (also called Mal), TRIF, and TRAM. TLR-ligand interaction causes the adaptor protein MyD88 to be recruited to the receptor complex, which in turn promotes its association with the IL-1R-associated kinases IRAK4 and IRAK1. During the formation of this complex, IRAK4 is activated, leading to the hyper-phosphorylation of IRAK-1, inducing the interaction of TRAF6 with the complex. The IRAK-4·IRAK-1·TRAF6 complex then interacts at the membrane with another preformed complex consisting of TAK1, TAB1, and TAB2. This interaction induces phosphorylation of TAB2 and TAK1, which then translocate together with TRAF6 and TAB1 to the cytosol. Engagement of TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR11 with their specific ligand results in recruitment of MyD88 to the receptor (24). However, TLR3 recruits TRIF after engagement with its ligands. Moreover, TLR4 is also able to recruit TRIF after stimulation, making it the only TLR that is able to utilize both MYD88 and TRIF dependent pathways. Due to the complex TLRs signaling these pathways are categorized into MyD88-dependent and TRIF-dependent pathways (43, 48, 49). These adapters activate other molecules within the cell, including certain protein kinases (TBK1, and IKKα) that amplify the signal, and ultimately lead to the induction or suppression of genes. Recruitment of these adaptors triggers the as activation of JNKs, p38 and ERK1/2 MAP kinases, which ultimately lead to the activation of transcription factors such as AP-1, NF-κB and IRFs. These

transcription factors induce the transcription of inflammatory cytokines, type 1 interferons and chemokines (44).

Mitogen-activated protein kinases and their signaling

The mitogen activated protein kinase (MAPK) signal transduction pathways are critical cascades involved in various physiological processes (50, 51). MAPK pathways are composed of three kinases that establish a sequential activation pathway comprising a MAP kinase kinase (MKKK), a MAPK kinase (MKK) and a MAP kinase (52, 53, 54). These are serine-threonine protein kinases that are activated by diverse stimuli. The stress-activated protein kinase (SAPKs) group of MAPKs includes members of the c-Jun NH2-terminal kinase (JNK) and p38 MAPK families (55, 56, 57). Three members of the JNK family have been identified, JNK1, JNK2 and JNK3. Each JNK gene is located on a different chromosome. In mice, JNK1 is on chromosome 14B, JNK2 is on chromosome 11B1.3, and JNK3 on chromosome 5. JNK3 is primarily expressed in the brain, heart, and testis but not in hematopoietic cells. JNK1 and JNK2 are expressed more ubiquitously; however their level of expression in T and B cells are very low prior to their activation. There are four isoforms of JNK1 (JNK1α1, JNK1α2, JNK1β1 and JNK1β2) described; however the relative contribution of these isoforms to overall JNK activity remains unknown (55, 58). Alternative splicing of JNK mRNA results in up to ten different protein products, varying in size from 46 KDa to 55 KDa (59, 60, 61). JNKs are activated by dual phosphorylation on Thr 183 and Tyr 185 by upstream MAPK kinase (MAPKK), specifically by SEK1 (also known as MKK4) and MKK7 (58). They then phosphorylate their respective substrates on serine or threonine residues. (62).

JNKs are considered the predominant protein kinase family that phosphorylates serine 63 and serine 73 within the trans-activation domain of c-Jun (63, 64). This phosphorylation potentiates c-Jun transcriptional activity by releasing the repressor complex histone deacetylase-3, which physically interacts with the N-terminal region of c-Jun (65). Additional substrates have been identified such as JunD and ATF2 that are members of the activator protein 1 (AP-1) transcription complex (66). AP-1 transcription factors are DNA binding heterodimers composed of Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1, Fra-2, Fosb2 and deltaFosB2), Maf, and ATF subunits (67). These transcription factors belong to the bZIP (basic-zipper) family and, as such, their dimerization is mediated by their leucine zipper domain. The recognition of the AP-1 site is mediated through the basic region (68). c-Jun is a major component of the AP-1 transcription factor. Its binding to specific sites on DNA results in activation and induction of many target genes involved in cell death, inflammation, cell differentiation, development and cancer (69, 70).

JNKs were originally identified by their ability to phosphorylate c-Jun in response to UV-irradiation; however, they are now recognized as critical regulators of other aspects of mammalian physiology such as cell proliferation, cell survival, cell death, DNA repair, and metabolism. In addition, JNK substrates have been identified in other cellular compartments, indicating that the action of JNK extends beyond transcriptional events, and may regulate other processes such as tumor development (71, 72). JNKs are important in a variety of different diseases such as infectious and autoimmune diseases as well as the initiation, progression and resolution of immune responses in vivo (73).

The activation of JNK during immune responses can be triggered by multiple cell surface receptors, including antigen receptors, TLRs, and cytokine receptors. The JNK pathway is important in the thymus during T-cell development and contributes to apoptosis during negative selection; it also plays a role during $CD4^+$ T cell effector function (74, 75, 76, 77). Although both JNK1 and JNK2 appeared to have an identical function, neither enzyme compensates for the deficiency of the other, indicating that they perform unique functions (54).

Methylation-controlled J protein (MCJ)

Molecular chaperones are a ubiquitous class of proteins that interact with short stretches of hydrophobic amino acids, typically exposed in partially unfolded proteins. Through such interactions, chaperones function in physiological processes that are essential for maintenance of normal cell activity and responses to different stimuli such as facilitating protein folding, protein translocation across membranes and remodeling of mulitmeric protein complexes (78). Hsp70 and J-proteins, which form different complexes, are among the most ubiquitous of the chaperones. Most eukaryotic and prokaryotic genomes encode multiple Hsp70s and J-proteins, suggesting their importance in cell function (78).

J-proteins contain a conserved 70 amino acid signature region; the J-domain, named after the *E-coli* protein, DnaJ. The DnaJ protein family is one of the largest with members with diverse cellular localization patterns such as the Golgi, ER and mitochondria that are critical for the de novo folding of nascent polypeptides, the interaction with signal transduction proteins, and the cell response to stress (79). The

DnaJ J-domain contains four α helices, with helices II and III forming a coiled-coil motif around the hydrophobic core. The most highly conserved amino acids of the J-domain, the histidine-proline-aspartate (HPD) tri-peptide located in the loop between helix II and III, is critical for the ATPase activity of these cochaperones (78). Methylation-controlled J protein (MCJ) is a newly identified member of the DnaJ protein family of cochaperones whose expression is primarily controlled by methylation (80). It is a Golgi compartment and mitochondrial-associated, type II transmembrane DnaJ protein. In addition to the DnaJ family, there are two other cochaperones that have been identified based on the presence of the Bag domain or the tetratricopeptide repeat clamp domain (81, 82). Cochaperones have a modular design in which a chaperone binding domain, such as DnaJ, is bound to other non-conserved sequences that can interact with specific proteins and mediate activities such as clathrin un-coating and cytoskeletal functions (83, 84). Moreover, DnaJ cochaperones also play a role in ubiquitin-dependent proteolysis by either tagging substrates for degradation or by facilitating the unfolding of proteins, which allows their proteolytic degradation (85).

MCJ has some unique features among the members of the DnaJ family. MCJ is comprised of 150 amino acids (aa), which is a rather small protein (16 to 17 kDa) compared to other members (~40 kDa). The DnaJ domain is located in the C-terminus of MCJ, while it is commonly located in the N-terminus in other DnaJ proteins (80). Moreover, a possible trans-membrane domain distinguishes MCJ from most other DnaJ proteins that are in the cytosol and interact with chaperones through the DnaJ domain, indicating that MCJ is atypical compared to other members of the DnaJ family (86).

The *mcj* gene is expressed in normal ovarian epithelial cells but not expressed or expressed at very low levels in many ovarian tumors and ovarian carcinoma cell lines (80). The region present within the first exon and first intron of MCJ gene constitutes a CpG island which can be hypermethylated, leading to its repression. High levels of Methylation in the CpG island correlate with poor responses of tumors to chemotherapy and over all poor survivor rate (87). While certain amount of interest has been given to the regulation of MCJ gene expression, no information about the biology and function of MCJ in immune cells is known. However, it has been shown that MCJ is involved in the regulation of ABCB1 pump gene expression and chemotherapeutic resistance in cancer cell lines, through the control of c-Jun protein stability.

Regulation of TNF expression and secretion

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine produced by several cell types, including macrophages. The cytokine was first discovered as an anti-tumor factor. Rapid production and secretion of TNF in response to inflammatory signals is central in development of strong innate and adaptive immune responses, by binding of TNF to its receptor TNFRI, and activating numerous signaling pathways that are important inactivation of immune responses such as MAP kinases and NF-κB and also by increasing vascular permeability which results in recruitment of immune cells; however, excessive and prolonged secretion of TNF can cause significant problems (88). Newly synthesized TNF quickly accumulates in the Golgi complex after stimulation and are transported to intermediate compartments before its delivery to the cell membrane. TNF biological activity is regulated by post-transcriptional events. There are two forms of

TNF: the full-length, type II trans-membrane protein, and the soluble form resulting from the porteolytic cleavage by the metaloprotease TNF-converting enzyme (TACE), that releases the active C-terminal portion from the cell surface (89, 90).

TACE is a member of the ADAM (A Disintegrin and A Metalloprotease) family of proteases. Similar to other ADAM proteins, TACE is a multi-domain type I trans-membrane protein, which contains a zinc-dependent catalytic domain and a disintegrin-cysteine rich sequence. TACE is constitutively expressed in most cells, with a long half time of 8 hours when translocated to the plasma membrane (90, 91). TACE activity is normally repressed by its physiological inhibitor, tissue inhibitor metalloproteinase 3 (TIMP3) that is a secreted protein. Among the four TIMPs, TIMP3 is the only one that binds to the extracellular matrix and contains an amino acid sequence (PFG) that uniquely inhibits TACE activity (90, 92). TIMP3 is induced by molecules involved in inflammation, such as PMA. TACE has emerged as a candidate to target TNF production for the treatment of many inflammatory diseases. Although important, little is known regarding the physiological and tissue-specific roles of TIMP-3-regulated and TACE-mediated TNF shedding (92, 93). The inducible deletion of c-*Jun and JunB* in epidermal cells in adult mice results in psoriasis-like inflammatory disease mediated by increased TNF secretion. These Jun proteins control TNF shedding in these cells by direct transcriptional activation of TIMP-3 (94). These and previous findings place Jun proteins as essential regulators of TNF production through the regulation of its transcription and surface shedding.

Pathogenic species (*B. burgdorferi sensu lato*)	Tick vector	Location
B. burgdorferi	I. *scapularis, I. pacificus*&*I. ricinus*	NE, NC & W USA, Europe
B. garinii	*I. ricinus, I. persulcatus*	Europe & Asia
B. afzelii	*I. ricinus, I. persulcatus*	Europe & Asia

Table 1.1 Pathogenic *B. burgdorferi,* their respective tick vectors and geographic distribution

B. burgdorferi sensu lato is comprised of the three genospecies of *B. burgdorferi* that cause human Lyme disease. There are other species of *Borrelia* that have been identified that are not able to cause infections or are minimally pathogenic.

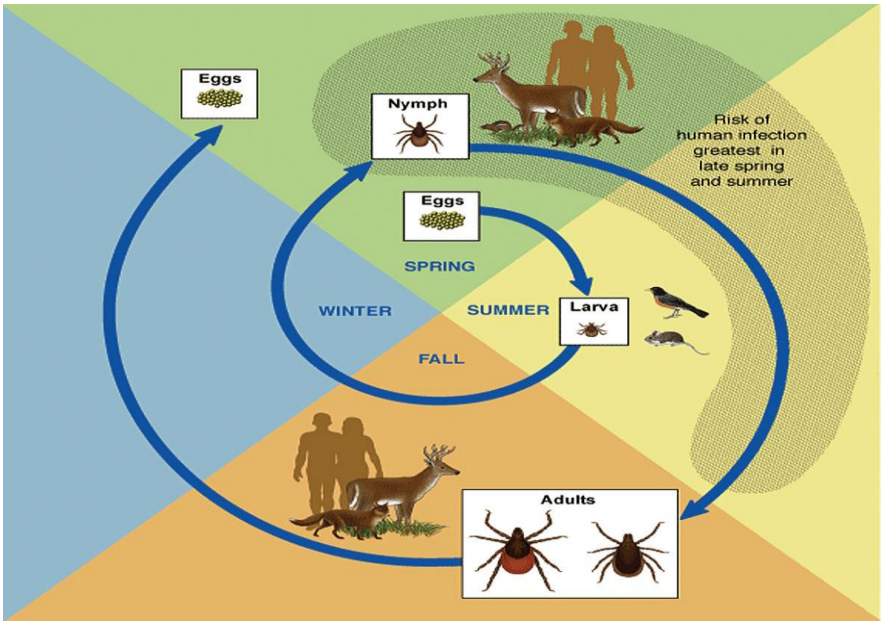

Figure 1.1 The lifecycle of *Ixodes scapularis*

The complete lifecycle of *Ixodes* ticks requires 2 years. Tick eggs are laid in the spring, and hatch as larvae in the summer. Larvae feed on small animals in the summer and early fall. The larvae may become infected with *B. burgdorferi* when feeding on these animals. Once a tick becomes infected, it can transmit the bacteria to other hosts. After this initial feeding, the larvae change into nymphs. Nymphs feed on rodents and other small mammals in late spring and early summer. It is at these times that nymphs can feed on humans and transmit the disease. Nymphs molt into adult ticks in the fall, feeding and mating on large animals such as deer. In early spring adult females lay their eggs on the ground completing their two-year lifecycle.

CHAPTER II

MATERIALS AND METHODS

Cells

JNK1- (24) and JNK2-deficient (68) C57BL/6 (B6) mice were used to purify splenic macrophages by positive selection, using a biotinylated antibody (Ab) against CD11b, $CD8^+$ and $CD4^+$ T cells (BD Pharmingen, La Jolla, CA). The purity of the cells, as determined by flow cytometry, was >85%. The macrophage cell line RAW264.7 (ATCC, Manassas, VA) was grown in RPMI medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum. MCJ-deficient and C57BL/6 (B6) mice were used to purify bone marrow derived macrophages.

All procedures that involved animals were in accordance with institutional guidelines for animal care at the University of Massachusetts at Amherst.

Plasmids, small interfering RNA (siRNA), and transfections

Plasmids containing a mutant (dominant-negative) version of human JNK1 (dnJNK), constructed by replacement of Thr183 and Tyr185 by Ala and Phe, respectively (57), or the luciferase gene downstream of 2x AP-1 (27) or 5x NF-κB (Stratagene, La Jolla, CA) response elements were used. The plasmids were transfected into 3 $x10^6$ to 5 x 10^6 RAW264.7 cells using DEAE-Dextran (Promega, Madison, WI) according to the manufacturer's protocol. The plasmid pBluescript SK(-) (SK) was used as a negative control in transfections with the plasmid containing dnJNK.siRNA targeting *jnk1* mRNA (Ambion, Austin, TX) was used to transfect 5 x 10^5 RAW264.7 cells using the siPort

Amine transfection agent (Ambion) following the manufacturer's instructions. Control siRNA (Ambion) containing a random mixture of oligonucleotides was used in parallel. After 48 h, the cells were assessed for *jnk1* mRNA by reverse transcriptase (RT) PCR (Table 1) and stimulated as described below.$1x10^6$ RAW264.7 cells were transfected with an siRNA plasmid targeting MCJ or an HA-MCJ plasmid over expressing MCJ, using Lipofectamin 2000 (Invitrogen) according to the manufacturer's protocol. Cells were incubated in the presence of hygromycin B (Invivogen, San Diego, CA) for two weeks and colonies were selected and further grown. siRNA targeting TIMP3 was used to transfect $1x10^6$ RAW264.7 and siMCJ cells using Lipofectamin 2000.

In vitro Stimulations

Low-passage *B. burgdorferi* N40 lysates were obtained from mid-log phase cultures by sonication. The protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA). The TRL1/TLR2 agonist *N*-palmitoyl-*S*-[2, 3-bis (palmitoyloxy)-(2*RS*)-propyl]-Cys-[*S*]-Ser-[*S*]-Lys_4trihydrochloride (PAM_3-CSK_4) was purchased from Invivogen (San Diego, CA). In vitro stimulations were performed using 10 µg/ml of a *B. Burgdorferi* lysate or 1 µg/ml of PAM_3-CSK_4.Live *B. burgdorferi* was grown in BSK-H medium (Sigma, St. Louis, MO), counted using dark filed microscopy and used at 10 or 25 MOI for stimulation of cells. The phosphorylation of STAT-1 in response to IFNγ stimulation of RAW264.7 cells was determined by stimulation with 100 ng/ml of recombinant murine IFNγ (R&D Systems, Minneapolis, MN) for 30 min. Phospho-Tyr^{701} and total STAT1 (Cell Signaling, Danvers, MA) were then detected by immunobloting.

JNK activity determination

Five million RAW264.7 or primary $CD11b^+$ cells were incubated with 10 µg/ml of a *B. burgdorferi* lysate for the indicated times. The cells were washed and lysed, and JNK activity was determined using a JNK activity assay kit (Cell Signaling) according to the manufacturer's protocol. Briefly, JNK was immunoprecipitated from the stimulated cell extracts with a c-Jun fusion protein bound to agarose. The immunoprecipitate was incubated in kinase buffer (25mM Tris (pH 7.5), 5mM b-Glycerophosphate, 2mM DTT, 0.1mM Na3VO4, and 10mM MgCl2) for 30 min at 30°Cand reaction was terminated with 3x SDS sample buffer. Phospho-c-Jun was then detected by immunoblotting using a rabbit phospho-c-Jun polyclonal Ab.

Cytokine ELISA

The levels of tumor necrosis factor (TNF) produced by *B. Burgdorferi-stimulated* primary macrophages and RAW264.7 cells were determined by capture enzyme-linked immunosorbent assay (ELISA), as described previously (95).

Antibodies

Anti-IκBα (Santa Cruz Biotechnology), anti-TLR1, and anti-TLR2 (Invivogen, San Diego, CA), Anti-TACE (eBioscience), Anti-Phospho-STAT1 (Cell signal), Anti-STAT (Cell signal), Anti-c-Jun (Santa Cruz Biotechnology), Anti-Jun D (Santa Cruz Biotechnology), Anti-c-Fos (Santa Cruz Biotechnology), Anti-ATF-2 (Santa Cruz Biotechnology), Anti-TNF (BD biosciences), Anti-MCJ (produced by Dr. Mercedes Rincón, UVM).

Western blotting

Immunoblotting was performed with stimulated and unstimulated cell extracts by lysing cells in lysis buffer (pH 7.4) containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM β-glycerol phosphate, 2.5 mM NaF, 2 mM sodium orthovanadate, 0.2 % sodium deoxycholate, 1 mM phenyl-methanesulfonyl fluoride (PMSF) and protease inhibitors, and protein levels were measured using the Bradford method. For TLR1 and TLR2 Western blots, a 7% polyacrylamide gel was used; for detection of MCJ and other low molecular weight proteins, a15% gel was used.

Flow cytometry and microscopy

Intracellular levels of TNF were determined by flow cytometry in siMCJ and RAW264.7 cells by incubating them with 25 MOI of *B. burgdorferi* and 1µl of Golgi plug for 6 hours followed by scraping and washing with PBS 1% FCS. Cells were incubated with cyotfix/cytoperm (BD Bioscience) for 20 min at 4^{o}C and resuspended in 50 µl of perm/wash solution and incubated with an TNF-Alexa 647 antibody or isotype control for 30 min at 4^{o}C. Cells were washed and resuspended in 500µl of PBS + 1% FCS. Cells were analyzed using FACS. Surface levels of TLR2 were determined in RAW264.7 and siMCJ cells by incubating $1x10^{6}$ cells in 100 µl of PBS + 1% FCS with an anti-TLR2 antibody (1/100 dilution) conjugated with PE for 30 min at 4^{o}C. The cells were washed and re-suspended in 500 µL of PBS + 1% FCS and analyzed in a LSRII flow cytometer (BD Bioscience). For microscopy $5x10^{4}$ cells were placed in a chamber slide over night, washed and fixed with 1% para-formaldehyde. The cells were permeabilized with PBS containing 0.01% triton-X100, blocked with 1% BSA and

stained with the appropriate antibody for 1 hour, then washed 5 times with PBS and stained with a secondary antibody conjugated with alexa fluor 568. Cells were analyzed by ApoTome microscopy.

Real-time and RT-PCR

Total RNA was isolated from primary macrophages and RAW264.7 cells using the TRIzol reagent (Invitrogen, Carlsbad, CA) accordingto the instructions of the manufacturer. PCR was performed to determine the expression of *tlr1*, *tlr2*, and the glyceraldehyde-3-phosphate dehydrogenase gene (*gapdh*) using 95°C denaturation, 55°C (*tlr1* and *tlr2*) or 60°C (*gapdh*) annealing, and 72°Cextension temperatures with the primers listed in Table 1.2.

Real-time RT-PCR to quantify *tlr1* and *tlr2* gene expression in dnJNK-transfected cells and BMMs from WT and MCJ-/- was performed using the primers listed in Table 1.2, and 2 µl of cDNA in a final volume of 20 µl. The reaction mixture contained SYBR green (Quanta Biosciences, Gaithersburg, MD) and ROX (as a reference dye). The reaction was performed at an annealing temperature of 55°C. Relative expression of the gene was determined by amplifying ß-actin (Applied Biosystems, Foster City, CA) and was referred to control (SK)-transfected cells, according to the following formula where C_T represents the threshold cycle for each gene:

$$Induction(n-fold) = 2^{-[(C_{Tlr1} - C_{Tactin})_{dnJNK} - (C_{Tlr1} - C_{Tactin})_{SK}]}$$

Real-time RT-PCR was also performed to quantify *mcj, tlr1, tlr2, tnf, tace* and *timp3* gene expression in RAW264.7, siMCJ and HA-MCJ cells using 2 µl of cDNA and the protocol described above with an annealing temperature of 60 °C.

Primers used in the study

Gene/target	Sequence*a*	Purpose
tlr1	5’-CGC AAA CCT TAC CAG AGT G-3’ 5’-GAC TGG CGT ATG CCA AAC TA-3’	RT-PCR
tlr1	5’-CTG GAG TCT GTT GTA GGA C-3’ 5’-GAC TGG CGT ATG CCA AAC TA-3’	Real-time PCR
tlr2	5’-AAG TGA AGA GTC AGG TGA TGG ATG TCG-3’ 5’-GCA GAA TCA ATA CAA TAG AGG GAG ACG C-3’	RT-PCR Real-time PCR
jnk1	5’-TGT GGA ATC AAG CAC CTT CAC TCT GCT G-3’ 5’-GCA AAC CAT TTC TCC CAT AAT GCA CCC-3’	RT-PCR
gadph	5'-CCA TCA CCA TCT TCC AGG AGC GAG-3’ 5’-CAC AGT CTT CTG GGT GGC AGT GAT-3’	RT-PCR
*tlr1*promoter	5’-AAG AGC TCC TGA GGT AAG GGG AAA CAG AG-3’ 5’-AAC CCG GGA AGA AAT TCA AGC ACT TCC TTG-3’	PCR
*tlr1*promoter	5’-AAT CAA CTT GTC AAA AAA GAC GCA TCC ATC CTG TAA CCA GCA CA-3’ 5’-TGT GCT GGT TAC AGG ATG GAT GCG TCT TTT TTG ACA AGT TGA TT-3’	Site-directed mutagenesis
tlr1-AP-1 (_230, _236)	5’-TAG TAA ACT GAC TGT AGT GA-3’	EMSA
tlr1-AP-1 (_471, _480)	5’-GCA CAT GAA TGA TCT TCC CT-3’	EMSA
tlr1-AP-1 (_502, _508	5’-AAA GAC GTG ATT AAC ATC CAT-3’	EMSA
AP-1 consensus	5’-CGC TTG ATG ACT CAG CCG GAA-3’	EMSA
tnfα	5’-AGC CCA CGT CGT AGC AAA CCA C-3’ 5’-ATC GGC TGG CAC CAC TAG TTG GT-3’	Real-time PCR
timp-3	5’-GGC CTC AAT TAC CG TAC CA -3’ 5’-CTG ATA GCC AGG GTA CCC AAA A -3’	Real-time PCR
tace	5_-TGG GAC ACA ATT TTG GAG CA -3’ 5’-CCT CCT TGG TCC TCA TTT GG-3’	Real-time PCR
mcj	5’-AAG TAA TCA CGG CAA CAG CAA GGA-3’ 5’-GAA GGC TGC CAG GCT TTT ATT-3’	Real-time PCR

Table 2.1 Sequences of the forward (F) and reverse (R) primers used for analysis of gene expression by PCR

Construction of *tlr1-luc* and *tlr1*(mut)-*luc* plasmids

The proximal DNA fragment corresponding to 1 kb of the human *tlr1* gene promoter region was sub-cloned upstream of the promoterless firefly luciferase (*luc*) gene in the vector pGL3-basic (Promega). The 1-kb promoter fragment was generated by PCR (Table 1) using genomic DNA isolated from HeLa cells as a template. The fragment was cloned into the pBAD-TOPO (Invitrogen) vector and sub-cloned using the *Sma*I and *Sac*I restriction sites into the pGL3 vector, generating the plasmid pGL3-*tlr1*-luc. The construct was sequenced across both junctions to confirm the nucleotide sequence and the right orientation. Empty pGL3 was used as a control. The pGL3-*tlr1*(*mut*)-luc plasmid, a derivative of pGL3-*tlr1-luc*with the AP-1-binding site deleted (nucleotides –502 to–508 relative to the transcription start site, Figure 1.10), was constructed using the QuikChange II XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The primers used for the deletion of the 7 bp are listed in Table2. The mutations were confirmed by sequencing.

Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Mini nuclear extracts were obtained as described previously (96) from $1x10^6$ RAW264.7 cells unstimulated or stimulated with10 µg/ml of a *B. burgdorferi* lysate for 16 h. Binding reactions were performed using 2 µg of nuclear protein in the presence of a specific ^{32}P end-labeled double-stranded oligonucleotide as described previously (96) in the absence or presence of unlabeled oligonucleotides. The oligonucleotides used in this study are listed in Table 1.2. Supershift/competition assays were performed using 1 µl of

anti-c-Jun (N), c-Fos (4), JunD (329), ATF-2 (C-19), and CREB (H-74) Abs (Santa Cruz Biotechnology).

Cytokine intracellular staining

Intracellular staining was performed by incubating RAW264.7 cells for 6 hours with 25 MOI of *B. burgdorferi* and 1µl of Golgi plug. The cells were stained for the surface marker F4/80, then washed and re-suspended in cytofix/cytoperm solution for 20 min to be fixed and permeabilized. Cells were stained in wash/perm solution for 30 min with anti-TFN antibody, washed and re-suspended in PBS + 1% FCS.

Bone marrow derived macrophages (BMDM)

Upon sacrifice, mouse legs were removed and tissue was eliminated from the pelvic and femoral bones. The bones were separated from the knee and dipped into 70% ethanol for 10 seconds to remove any contaminants. The ends of each bone were cut and the bone marrow was extracted using 25g needles and RPMI medium. The cells were eliminated of red blood cells and resuspended in RPMI supplemented with 20% of L292 conditioned medium. The cells were incubated 6 to 8 days in conditioned media, washed, and incubated 24 h in normal RPMI prior to the experiments.

TACE activity

To measure cellular TACE activity, $1x10^6$ cells were washed and resuspended in 400 µl of PBS + 1% FCS. 100 µl of cells were incubated in a glass cuvette with 10µM of TNF FRET (21) peptide (ANASPEC San Jose, CA). Cleavage of the FRET peptide by TACE results in the release of fluorescence that was measured in a TD20/20 luminometer

equipped with a fluorescent module (Turner Biosystems). Background florescence was measured at time zero just after addition of the FRET peptide with subsequent measurements at different time points after incubation at 37 °C. Florescence measurements were related to time zero measurements. Specific TACE activity was determined by subtraction of parallel measurements in cells treated with the inhibitor TAPI-2 (ENZO Life Sciences, Plymouth Meeting, PA).

Statistical analyses

Data are presented as the means and standard errors of at least three independent experiments or means and standard deviations for experiments performed in triplicate. The means of independent experiments were compared with two-way analysis of variance, followed by Bonferroni posttests, using the software Prism version 4.0. The means were considered statistically different when p was ≤ 0.05.

CHAPTER III

RESULTS

The role of JNK1 in macrophages and its involvement in the mechanism that regulates *tlr1* gene transcription

JNK activity regulates TNF production in response to *B. burgdorferi*

The role played by the JNK pathway in phagocytic cell responses to pathogens mediated by TLRs is poorly understood. To address whether the interaction between *B. burgdorferi* antigens and phagocytic cells results in the activation of JNK, we stimulated the macrophage cell line RAW264.7 with a *B. burgdorferi* lysate. The cells were stimulated for 0, 20, 40, 60, 80 and 120 minutes and the activation of JNK was analyzed by Western blot of the phosphorylated form of c-Jun, a substrate of JNK. JNK activity was evident after 40 minutes of stimulation and decayed after 80 minutes (Fig. 3.1A). Total levels of c-Jun were also measured to ensure equal protein loading. We also analyzed JNK activity in primary macrophages isolated form C57Bl/6 mice. Purified splenic macrophages were stimulated with 10 μg/ml of a *B. burgdorferi* lysate for 40 minutes and JNK activity was analyzed by Western blotting. Activation of primary macrophages also resulted in the activation of JNK (Fig. 3.1B). These data indicated that the interaction of spirochetal antigens with phagocytic cells results in the activation of the JNK MAP kinase pathway.

We then addressed the involvement of JNK activity in macrophage cytokine production in response to *B. burgdorferi* antigens. RAW264.7 cells were transfected with a dominant negative form of JNK1 (dnJNK1) (97), and stimulated with a *B. burgdorferi*

lysate or the TLR1/TLR2 agonist PAM_3-CSK_4, and the supernatants were analyzed 16 hours after stimulation for the presence of TNF. Repression of JNK activity resulted in the reduction of TNF production compared to control transfected cells (Fig. 3.2A). We also purified primary macrophages from spleens of B6 mice; they were stimulated with a *B. burgdorferi* lysate or PAM_3-CSK_4 in the presence of increasing concentrations of the JNK1 specific inhibitor SP600125 (98) (Fig. 3.2B), showing specific contribution of JNK1activity to cytokine production by macrophage in response to *B. burgdorferi*. These results indicate that JNK1 activity is involved in *B. burgdorferi*-induced TNF production by macrophages.

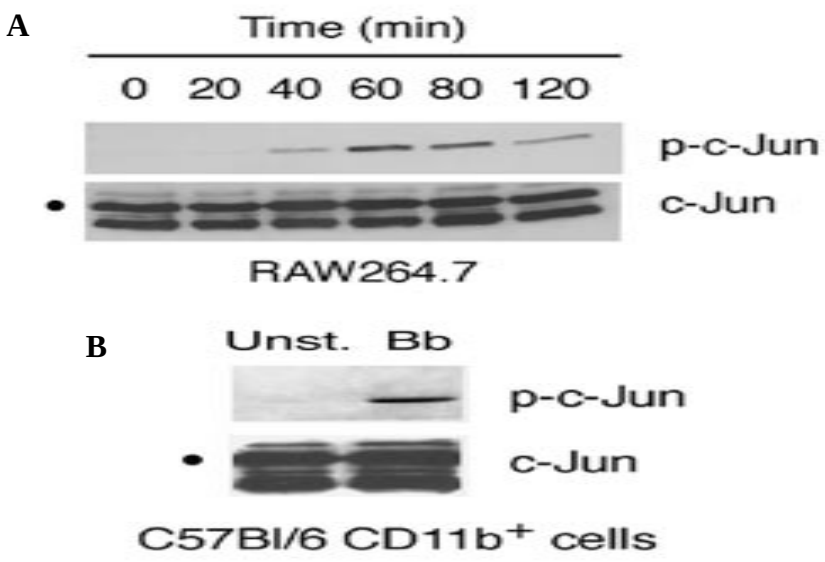

Figure 3.1 JNK activity in RAW264.7cells and primary macrophages

(A) RAW264.7cells (B) and purified splenic $CD11b^{+}$cells were stimulated with a *B. burgdorferi* lysate for the indicated times (top) or 40 min (bottom) prior to assaying JNK activity *in vitro* with c-Jun as a substrate. Phospho-c-Jun was then detected by immunoblotting.

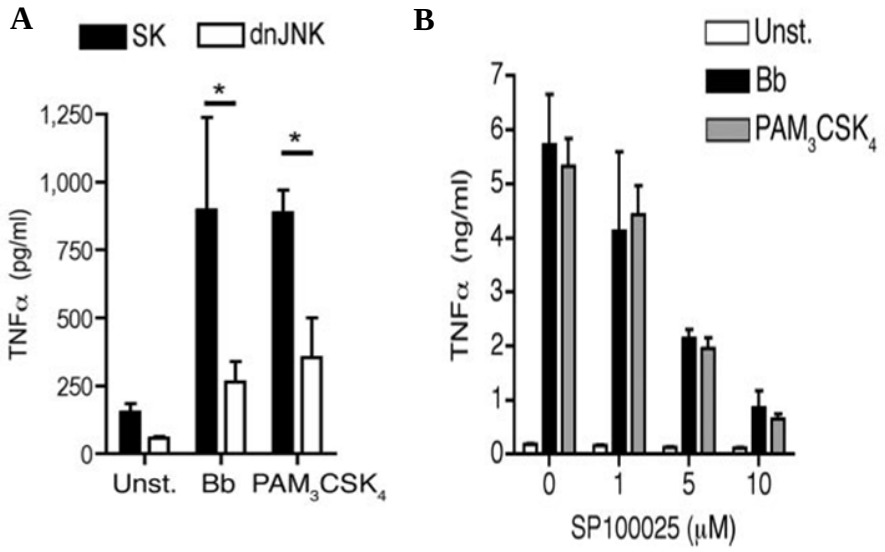

Figure 3.2 JNK activity regulates TNF production in response to *B. burgdorferi*

(A) RAW264.7 cells were transfected with a plasmid containing dnJNK1 or a plasmid control (SK) and stimulated with a *B. burgdorferi* lysate (Bb) or PAM_3-CSK_4 for 16 h. TNF levels in the stimulation supernatants were then quantified by ELISA. The results shown are the average plus standard error (SE) of three independent experiments.*****, $p < 0.001$ and $p < 0.01$ for *B. burgdorferi* and PAM_3-CSK_4 stimulation, respectively. (B) RAW264 cells were stimulated with 10 µg/ml of a *B. burgdorferi* lysate (Bb) or 1 µg/ml of PAM_3-CSK_4 in the presence of increasing concentrations of the JNK inhibitor SP100625.TNF was quantified in the stimulation supernatants after 16 h.

TLR1/2-mediated responses are dependent on JNK activity

Toll-like receptors (TLRs) play critical roles during the initiation of innate immunity and the development of specific cell-mediated immune responses (24). TLR engagement results in the activation of the mitogen-activated protein p38 and c-Jun N-terminal kinases (JNK) (99). It has been shown that the interaction of *B. burgdorferi* lipoproteins with immune cells increase the expression of TLR1 and TLR2 (100). To substantiate the contribution of JNK activity on TLR1/TLR2-induced signaling events in macrophages, we analyzed the activation of JNK-dependent and independent transcription factors. JNK activity results in the phosphorylation of c-Jun and the formation of AP-1 complexes (54). To assess the contribution of JNK on macrophage responses to *B. burgdorferi* mediated by c-Jun, RAW264.7 cells were co-transfected with a plasmid containing the luciferase gene under the influence of AP-1 or NF-κB response elements plus a plasmid containing dnJNK or a control plasmid. Cells were stimulated with a *B. burgdorferi* lysate and luciferase activity was measured after 16 hours. As expected, the repression of JNK activity during *B. burgdorferi* stimulation resulted in decreased AP-1 transcriptional activity (Fig. 3.3 A). As a control, we also analyzed the activation of JNK-independent signaling pathways in response to *B. burgdorferi* antigens by assessing NF-κB transcriptional activity. Surprisingly, the repression of JNK activity also resulted in reduced NF-κB transcriptional activity (Fig. 3.3B). No reports have associated JNK activity and the activation of NF-κB. Since the repression of JNK activity during stimulation with TLR ligands results in lower TNF production, and this cytokine activates NF-κB (101), we analyzed I-κBα degradation, by transfecting RAW264.7 cells with dnJNK or a control plasmid and stimulation with a *B. burgdorferi* lysate at time

points in which the production of the cytokine is not evident. Cells transfected with dnJNK plasmid resulted in decreased degradation of I-κBα compared to controls (Fig. 3.4A). The results were confirmed by band quantitation of the I-κBα band using the software ImageJ (Fig. 3.4B).

In order to assess whether the repression of JNK in phagocytic cells results in both TLR-dependent and independent cell responses, we determined the effect of JNK repression in response to IFNγ-induced signals. RAW264.7 cells were transfected with dnJNK or a control plasmid and stimulated with rmIFNγ for 30 minutes. Phospho-STAT1 and total STAT1 levels were measured by immunoblot. The phosphorylation of STAT1 induced by IFNγ was not affected by the presence of the dnJNK-containing plasmid inRAW264.7 cells (Fig. 3.5), indicating that JNK affects TLR1/2-mediated, but not TLR-independent responses in macrophages.

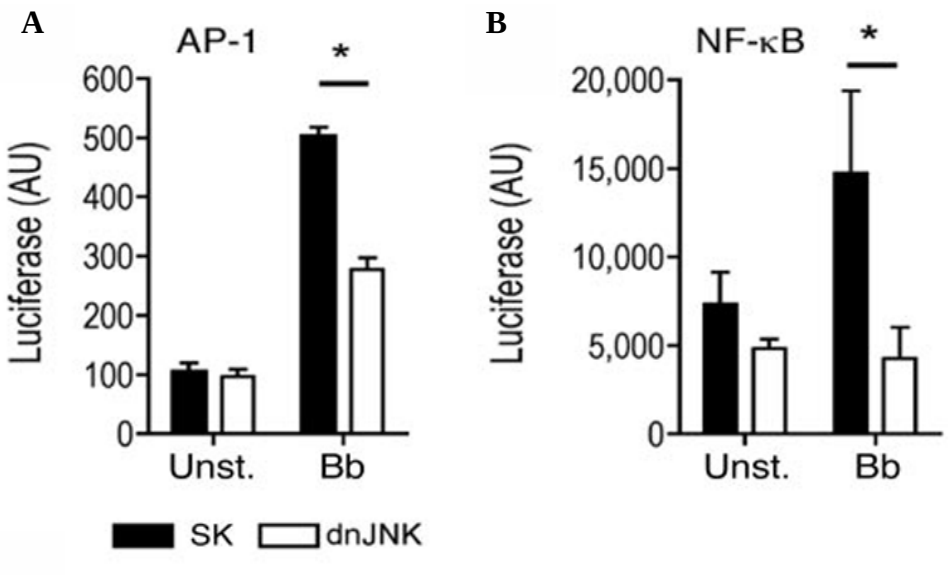

Figure 3.3 JNK regulates macrophage responses to *B. burgdorferi*

RAW264.7 cells were co-transfected with a plasmid containing the *luciferase* gene under the influence of AP-1 (A) or NF-κB (B) response elements plus a plasmid containing the dnJNK form or a plasmid control (SK). The cells were then stimulated with a *B. burgdorferi* lysate (Bb) for 16 h, and luciferase activity was assayed. The results shown are the average standard error of four and three independent experiments, respectively. *****, $p < 0.001$ (AP-1-luc) and $p < 0.05$ (NF-κB-luc).

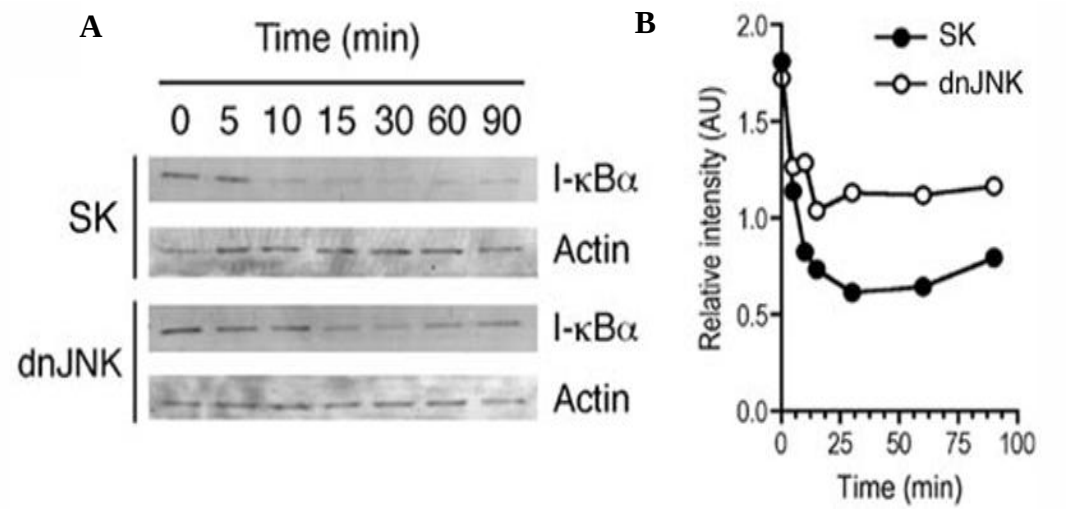

Figure 3.4 Inhibition of JNK results in decreased degradation of IκBα

RAW264.7 cells were transfected with a plasmid containing the dnJNK form or a plasmid control (SK) and stimulated with a *B. burgdorferi* lysate for the indicated times. The cells were then lysed and assessed for IκBα content by immunoblotting (A). Band quantitation was performed with the Java-based software ImageJ (B). The experiment shown is representative of three performed.

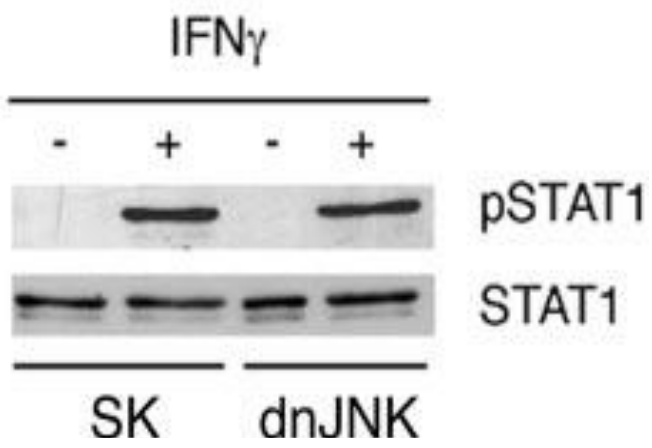

Figure 3.5 JNK does not affect TLR independent responses

RAW264.7 cells were transfected with a plasmid containing the dnJNK form or a plasmid control (SK) and stimulated with recombinant murine IFN-γ for 30 min. Phospho-STAT1 and totalSTAT-1 levels were determined by immunoblotting.

JNK1 regulates the expression of the *tlr1* gene

The interaction of TLRs with their specific ligands leads to the activation of different cell signaling components. Lipidated proteins of *B. burgdorferi* bind heterodimers of TLR-1 and TLR-2, leading to the up regulation of inflammatory genes and surface receptors. To assess the hypo-responsiveness of these cells to *B. burgdorferi* antigens and to test whether JNK is involved in the regulation of expression of the components involved in TLR-mediated responses, we analyzed *t*lr1 and *tlr2* levels in macrophages. cDNA was generated from RAW264.7 cells transfected with dnJNK and control plasmids and expression levels of *tlr1* and *tlr2* were assessed by PCR. Cells transfected with dnJNK showed lower expression levels of *tlr1* compared to controls; however the presence of dnJNK did not have any effect on *tlr2* gene expression (Fig. 3.6A). Furthermore, we determined *tlr1* and *tlr2* gene expression using qPCR in RAW264.7 cells transfected with the dnJNK or control plasmids. The level of *tlr1* gene expression was greatly reduced in cells transfected with dnJNK while no difference was observed in the level of *tlr2* gene expression (Fig. 3.6B). To assess TLR1 protein levels, we transfected RAW264.7 cells with the dnJNK and control plasmids. Protein levels were determined by Western blotting and protein input was assessed by immunobloting with anti-Actin. Correlating with gene expression levels, TLR1 protein levels were reduced in cells with repressed JNK activity compared to control cells, while TLR2 protein levels were unaffected by repressed JNK activity (Fig. 3.6C).

To further demonstrate the regulation of *tlr1* gene expression by JNK, we analyzed the level of expression of both *tlr1* and *tlr2* genes in primary macrophages. Macrophages were purified from spleens of WT, JNK1 and JNK2-deficient mice. As expected, *tlr2* mRNA levels were not affected by the lack of either JNK1 or JNK2, while *tlr1* mRNA levels were reduced in JNK1-, but not JNK2-deficient macrophages (Fig. 3.6D). Furthermore, we transfected RAW264.7 cells with siRNA specific for *jnk1*or a control siRNA mixture (siNC) and extracted RNA to determine the mRNA levels of *tlr1* and *tlr2*. Equal input was determined by amplification of glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) mRNA. The silencing of JNK1-encoding mRNA in RAW264.7 cells resulted in lower *tlr1* gene expression compared to controls (Fig. 3.6E), suggesting that JNK1 activity specifically regulates the expression of TLR1.

To assess the contribution of the JNK isoforms on TNF production in the presence of *B. burgdorferi*, we stimulated JNK1 and JNK2-deficient $CD11b^+$ cells with *B. burgdorferi* lysate for 16 hours and the supernatants were analyzed by ELISA. Both JNK1 and JNK2 deficient macrophages produced lower levels of TNF, compared to wild-type macrophages (Fig. 3.7A and B), indicating that JNK1 and JNK2 regulate the production of TNF through different mechanisms that does not involve the regulation of TLR1 in the case of JNK2. Furthermore, RAW264.7 cells were transfected with siRNA against JNK1 or a control siRNA and stimulated with a *B. burgdorferi* lysate or PAM_3CSK_4 for 16 hours and supernatants were assessed for TNF by ELISA. The cells transfected with siJNK1 produced significantly lower levels of TNF compared to control cells (Fig. 3.7C). In correlation, the induction of TNF by the TLR4, TLR9, and TLR5

agonists lipopolysaccharide, poly(dI-dC), and flagellin, respectively, was lowered in cells transfected with the plasmid containing dnJNK (Fig. 3.7D).

To demonstrate whether JNK1 regulates TLR1/2-mediated responses solely through the control of TLR1 expression; RAW264.7 cells were co-transfected with a plasmid that drives the constitutive expression of TLR1 (UNO), plus a plasmid containing the dnJNK form or a control plasmid. Twenty four hours after transfection, the cells were stimulated with a *B. burgdorferi* lysate or PAM_3CSK_4 and TNF levels in the supernatants were measured. The ectopic expression of TLR1 prevented the inhibition of TNF production in the presence of the dnJNK plasmid in response to *B. burgdorferi* antigens and PAM_3CSK_4 (Fig. 3.8), confirming that JNK1 controls TLR1/2-mediated signals in macrophages solely by regulating the expression of TLR1.

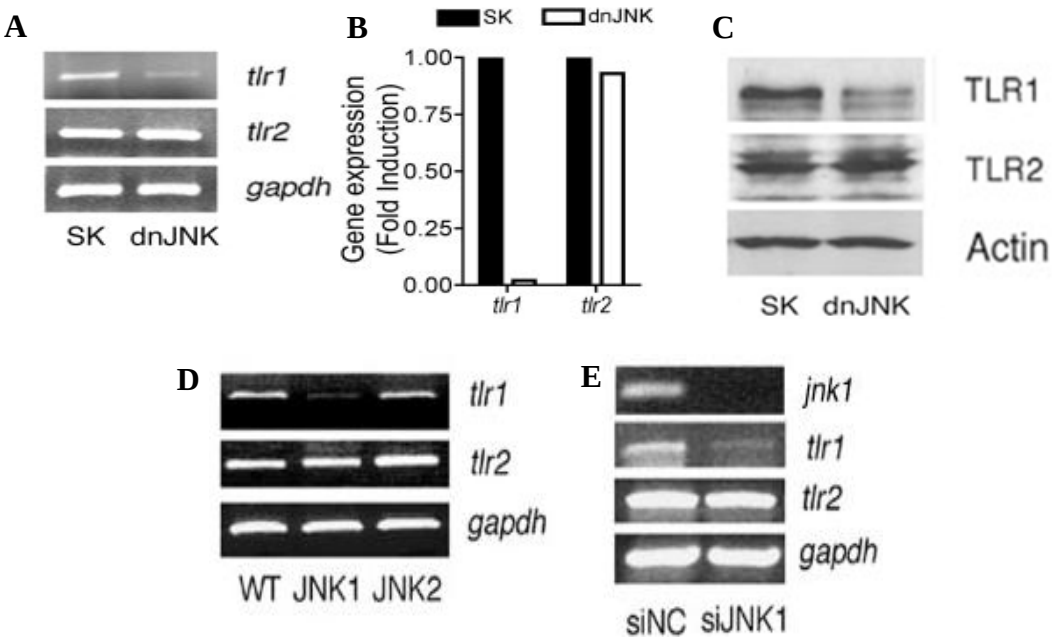

Figure 3.6 JNK1 regulates the expression of the *tlr1* gene and TLR1 protein

(A) RAW264.7 cells were transfected with a plasmid containing a dnJNK form or a plasmid control (SK), and total RNA was extracted and assessed by RT-PCR or (B) real-time RT-PCR for the expression of *tlr1* and*tlr2* mRNAs. Equal input of RNA was assessed by the amplification of the housekeeping gene *gapdh*. (C) The cells were also used to determine the levels of TLR1 and TLR2 by Western blotting. Protein input was assessed by immunobloting with an anti-actin Ab. (D) $CD11b^{+}$ cells were purified from B6 (WT) and JNK1- and JNK2-deficient mice. RNA was extracted and subjected to RT-PCR for the expression of *tlr1*and *tlr2*. RNA input was assessed by the amplification of *gapdh* mRNA. (E) RAW264.7 cells were transfected with JNK1 siRNA or an siRNA control (siNC) and used to extract RNA to determine the mRNA levels of *jnk1*, *tlr1*, and *tlr2*. Equal input was determined by amplification of *gapdh* mRNA.

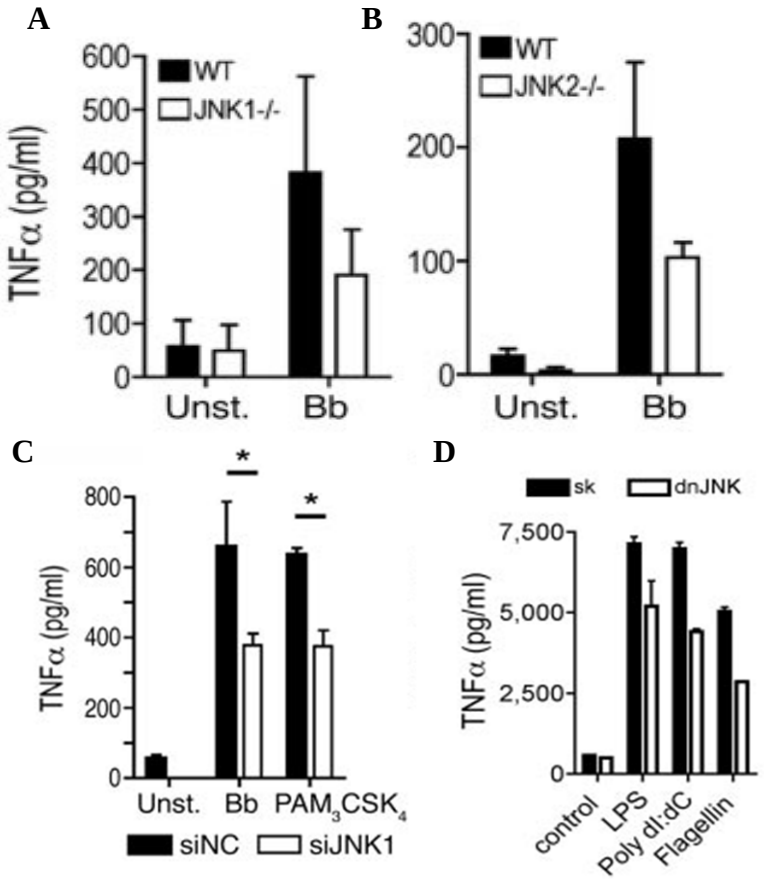

Figure 3.7 Contribution of the JNK isoforms to TNF production in response to *B. burgdorferi*

(A) (B) Purified $CD11b^+$ cells from B6 (WT) and JNK1- and JNK2-deficient mice were unstimulated (Unst.) or stimulated with a *B. burgdorferi* lysate (Bb). The levels of TNF were determined in the stimulation supernatants after 16 h. (C) RAW264.7 cells were transfected with siRNA oligonucleotides specific for JNK1 or control oligonucleotides (siNC). Forty-eight hours later, the cells were stimulated for 16 h with a *B. burgdorferi* lysate (Bb) or PAM_3-CSK_4 and assessed for TNF by ELISA. The results represent the average plus standard error (SE) of three independent experiments. *****, $p < 0.05$. (D) dnJNK1-transfected RAW264.7 cells were stimulated with lipopolysaccharide (LPS), poly (dI-dC), and flagellin, and TNF was measured16 h later.

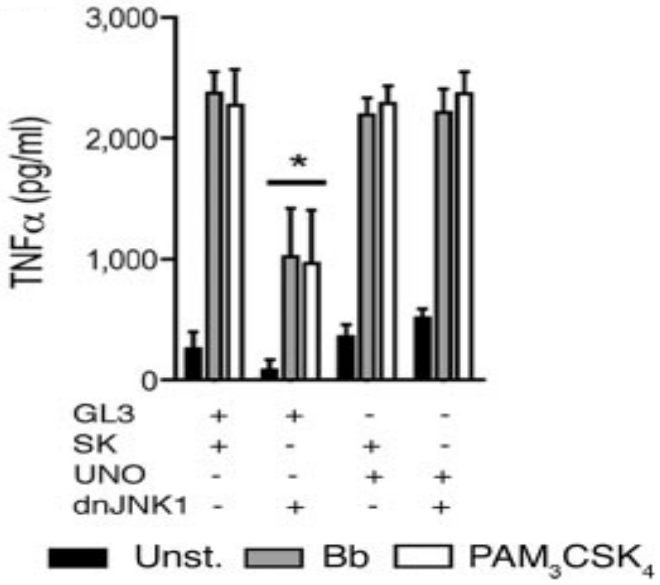

Figure 3.8 JNK1 regulates TLR1/2-mediated responses solely through the control of TLR1 expression

RAW264.7 cells were co-transfected with a plasmid encoding TLR1 under the influence of a constitutively expressed promoter (UNO) or a control plasmid (GL3) plus a plasmid containing the dnJNK form or the empty plasmid (SK). All cells were transfected with the same amount of plasmid. The cells were then stimulated with a *B. burgdorferi* lysate (Bb) or PAM_3CSK_4. TNF levels in the supernatants were determined 16 h after stimulation. The results shown represent the average plus SE of three independent experiments. *****,$p< 0.05$ compared to control-, UNO plus control-, or UNO plus dnJNK-transfected cells stimulated with *B. burgdorferi* and PAM_3-CSK_4.

JNK1 regulates *tlr1* promoter activity

To further demonstrate that JNK activity regulates *tlr1* gene transcription, we cloned the proximal 1 kb promoter region of the *tlr1* gene (Fig. 3.9) upstream of a promoterless luciferase gene. RAW264.7 cells were co-transfected with a plasmid containing 1 kb proximal *tlr1* promoter, and the dnJNK plasmid or a plasmid control, cells were then stimulated with a *B. burgdorferi* lysate, PAM_3CSK_4 or left unstimulated and Luciferase activity was measured. The transfection of RAW264.7 cells with this construct resulted in basal levels of promoter activity while the activity of the promoter was enhanced by the stimulation of the cells (Fig. 3.10A). The co-transfection of RAW264.7 cells with the dnJNK-containing plasmid resulted in the repression of promoter activity (Fig. 3.10A), confirming that JNK is involved in the regulation of TLR1 activity. The increased Luciferase expression correlated with augmented TLR1 protein levels in RAW264.7 cells that had been stimulated with a *B. burgdorferi* lysate (Fig. 3.10B).

Three putative AP-1 binding sites were identified that could serve as potential AP-1 binding sites in the *tlr1* promoter (Fig. 3.9). Double stranded oligonucleotides that spanned these regions were used for EMSAs (Table 2.1). From these three sites only the oligonucleotide based on the sequence found at -502 to -508 showed binding of nuclear extract preparations of unstimulated and *B. burgdorferi* stimulated RAW264.7 cells (Fig. 3.11A). We observed two binding complexes at this site. The lower complex increased in *B. burgdorferi*-stimulation RAW264.7 compared to unstimulated cells and was competed when the binding reaction contained anti-c-Jun and anti-c-Fos Abs (Fig. 3.11B). This complex was also competed by unlabeled double stranded oligonucleotides

corresponding to the AP-1 consensus binding site and coincided with the complex obtained with a consensus AP-1 binding site oligonucleotide (Fig. 3.11C). The presence of ATF-2 and JunD antibodies inhibited the formation of both complexes, while antibodies to CREB did not compete (Fig. 3.11B). These results suggested that this region serves as a docking area for two complexes that contain ATF-2 plus JunD, and c-Jun plus c-Fos. These data also suggested that the upper complex is involved in the constitutive expression of the *tlr1* gene, while the lower complex is formed in response to stimulation with *B. burgdorferi* antigens. Since ATF-2 and JunD are substrates of JNK (96), this interpretation is in agreement with the control of basal levels of *tlr1* gene expression by JNK. Overall, these data demonstrated the presence of functional AP-1 binding sites in the proximal promoter region of the *tlr1* gene.

We further tested the contribution of the -502 to -508 binding site to the regulation of *tlr1* gene expression mediated by JNK. We first deleted this binding site from the *tlr1* promoter region, and then cloned the deletion mutant upstream of the *luciferase* gene generating the plasmid *tlr1*Δ(-502,-508) [*tlr1* (mut)]-luciferase. RAW264.7 cells were then co-transfected with plasmids containing *tlr1*-luc or *tlr1* (mut)-luc with dnJNK or a control plasmid. Cells were rested for 48 hours and stimulated with a *B. burgdorferi* lysate or PAM_3CSK_4, and luciferase activity was measured. The deletion of the AP-1 binding site on the *tlr1* proximal promoter region resulted in decreased promoter activity that was not enhanced by stimulation (Fig. 3.12). Furthermore, compared to the wild-type promoter, the mutant form was not affected by the co-expression of the dominant negative form of JNK (Fig. 3.12). Overall, these data demonstrate that JNK1 activity regulates the expression of TLR1 in macrophages.

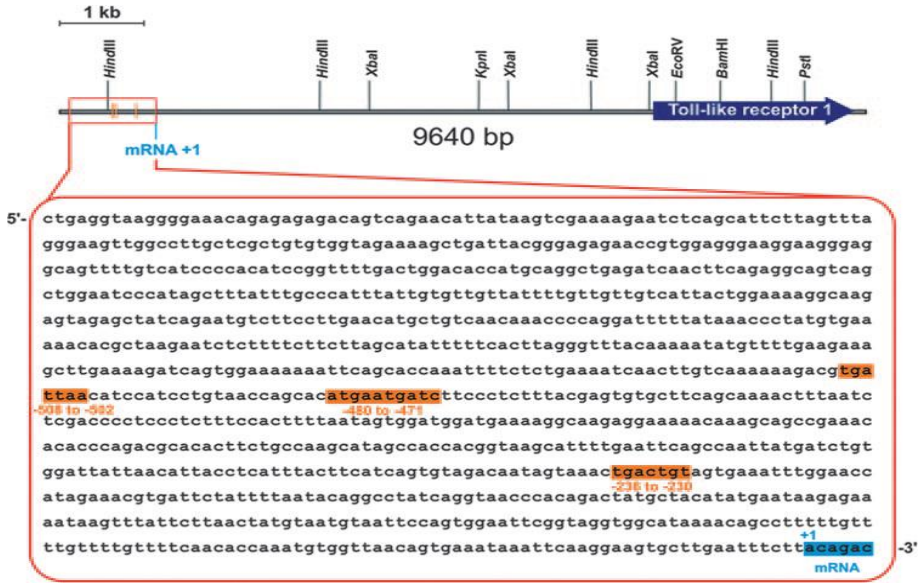

Figure 3.9 Structure of the human *tlr1* gene

The 1-kb proximal promoter and the gene encoding TLR1 are shown. The proximal promoter sequence is shown in the magnified box, with the three putative AP-1 binding sites highlighted. The sequence corresponds to nucleotides 5956071to 5955050 of contig NT016297 (NCBI/NIH).

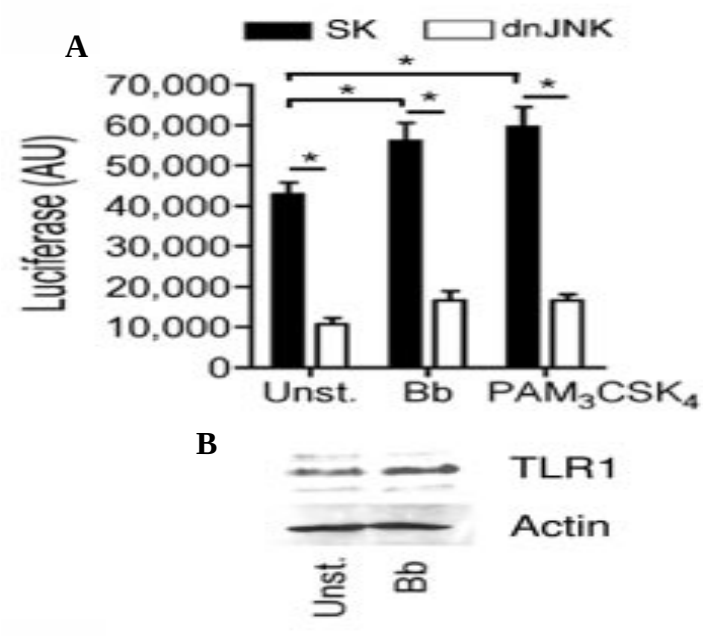

Figure 3.10 JNK1 regulates *tlr1* promoter activity

(A) RAW264.7 cells were co-transfected with a plasmid containing the luciferase gene downstream of the 1-kb proximal *tlr1* promoter and the dnJNK plasmid or a plasmid control (SK) and stimulated with a *B. burgdorferi* lysate (Bb) or PAM_3-CSK_4or left unstimulated (Unst.). Luciferase activity (AU, arbitrary units) was measured after 16 h of stimulation. The data presented correspond to the average plus standard deviation of triplicate determinations of one of four experiments performed. *****, $p < 0.05$. (B)Western blot of RAW264.7cells stimulated with 10 µg/ml of a *B. burgdorferi* lysate for 16 h using anti-TLR1 Ab. Equal protein input was assessed with anti-Actin Ab

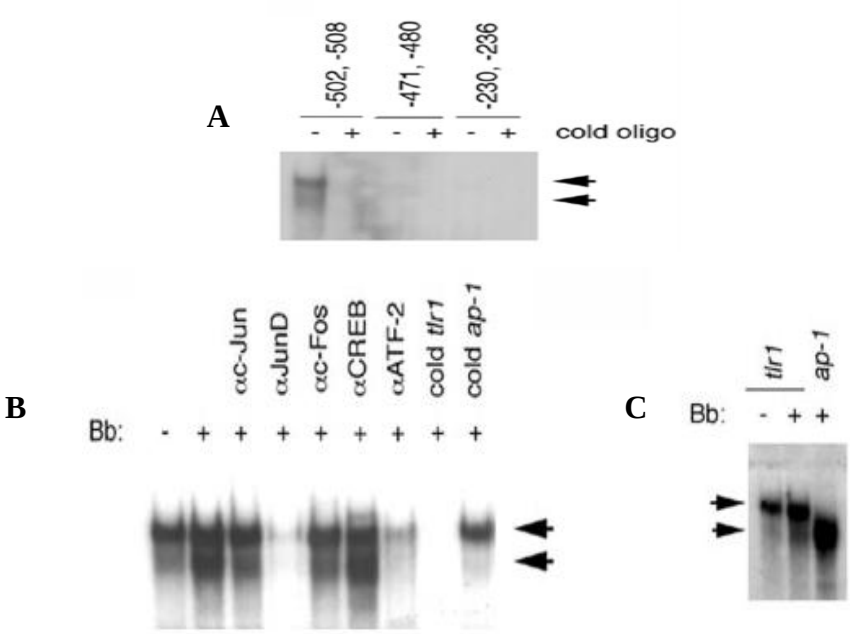

Figure 3.11 Regulation of *tlr* promoter binding is mediated by JNK

(A) EMSA showing nuclear extracts binding to oligonucleotides (oligo) containing putativeAP-1 binding sites in the proximal 1-kb *tlr1* promoter. The reactions were performed in the absence or presence of an excess (100x) of unlabeled (cold) oligonucleotides. (B) EMSA of nuclear extracts of RAW264.7 cells unstimulated and stimulated with a *B. burgdorferi* lysate for 30 min. The reactions were performed in the absence or the presence of Abs against c-Jun, JunD, c-Fos, CREB, and ATF-2 and in the absence or presence of unlabeled (cold) oligonucleotides corresponding to the *tlr1* promoter or the AP-1 consensus binding sequence. The arrows indicate the constitutive and *B. burgdorferi*-enhanced complexes formed. (C) EMSA of *tlr1*-derived and consensus AP-1 binding oligonucleotides with nuclear extracts from unstimulated or *B. burgdorferi*-stimulated RAW264.7 cells

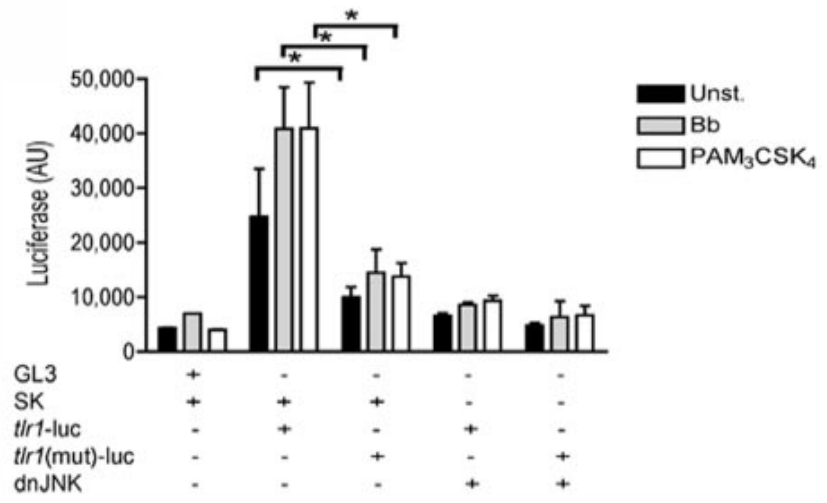

Figure 3.12 Contribution of the 502 to 508 binding site to the regulation of *tlr1*gene expression mediated by JNK

RAW264.7 cells were co-transfected with plasmids containing *tlr1*- or *tlr1*Δ(*502,508*) [*tlr1*(*mut*)]-*luciferase* and a plasmid containing dnJNK or a plasmid control (SK). After48 h, the cells were stimulated with a *B. burgdorferi* lysate (Bb) orPAM_3-CSK_4or left unstimulated (Unst.). Luciferase activity was measured 16 h post stimulation.

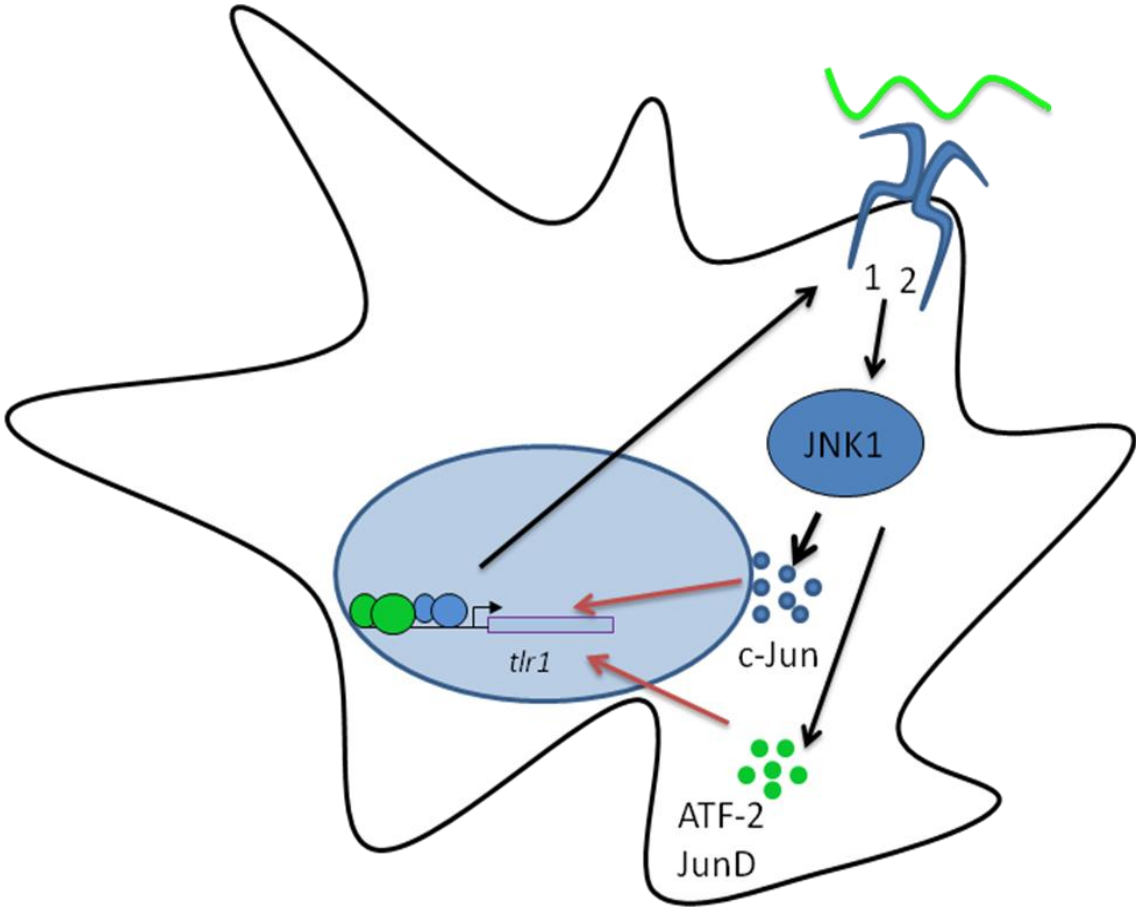

Figure 3.13 JNK1 regulation of *tlr1* gene expression model

The contribution of MCJ to the regulation of macrophage responses to *B. burgdorferi*

MCJ expression in innate immune cells results in regulation of c-Jun levels

MCJ is a co-chaperone that belongs to the DnaJ family. The *mcj* gene is expressed in normal ovarian epithelial cells but not expressed or expressed at very low levels in many ovarian tumors and ovarian carcinoma cell lines (80). MCJ regulates the expression of ABCB1 pumps mediated by c-Jun in the MCF-7 breast cancer cell line, and loss of MCJ is involved in cancer resistance to chemotherapeutic drugs. Regulation and involvement of MCJ in these processes has been linked directly to its ability to regulate the stability of the c-Jun protein. However, the function of MCJ in innate immune cells, specifically in macrophages, is unknown. Since JNK regulates innate immune cell responses to *B. burgdorferi* through c-Jun, and MCJ regulates c-Jun protein levels in cancer cells, we were interested in determining the possible functions of MCJ in macrophages.

We first determined whether MCJ is expressed in macrophages. MCJ levels were determined by purifying $CD11b^+$, $CD4^+$ and $CD8^+$ T cells from the spleens of wild type C57/B6 mice. Base on the information obtained from our collaborator (Dr. Mercedes Rincón, UVM) $CD4^+$ and $CD8^+$T cells were used as negative and positive controls, respectively and compared to $CD11b^+$ cells (macrophages). The cells were lysed and Western blot was performed using 15% SDS-PAGE to detect MCJ. MCJ protein was detected in $CD11b^+$ cells and at higher levels in $CD8^+$ T cells; however MCJ protein was not detectable in $CD4^+$ T cells (Fig. 3.14A). We next performed ApoTome fluorescence microscopy to detect MCJ. RAW264.7 cells were grown in chamber slides, fixed,

permeablized and stained with a rabbit anti-mouse MCJ antibody followed by anti rabbit IgG conjugated to Alexa Fluor-568. MCJ was readily detected in $CD11b^+$ and $CD8^+$ T cells; however, MCJ was detected at extremely low levels in $CD4^+$ T cells probably due to other contaminating cells in the preparation (Fig. 3.14B). RNA was also extracted from these cells and qRT-PCR was performed using *mcj*-specific primers. The expression levels of *mcj* correlated with the protein levels and microscopy results (Fig. 3.14C). These data demonstrated that MCJ is expressed in macrophages. To assess the role that MCJ plays in macrophages, we first generated stably transfected RAW264.7 cells with a plasmid expressing siRNA against MCJ (siMCJ), or a plasmid encoding MCJ with Hemagglutinintag (HA) (HA-MCJ). We first assessed whether cells with repressed MCJ or cells that over-express the cochaperone showed variation in c-Jun protein levels, similarly to what has been described in cancer cells. RAW264.7 and siMCJ cells were lysed and Western blot was performed for c-Jun. c-Jun protein levels were elevated in cells with repressed MCJ compared to control cells (Fig. 3.15A). In correlation, cells over-expressing MCJ had lower levels of c-Jun compared to control cells (Fig. 3.15B). We confirmed these data by generating Bone marrow derived macrophages (BMDM) from WT and MCJ-deficient mice. MCJ-deficient cells contained higher levels of c-Jun compared to WT controls (Fig. 3.15C). Equal loading for these experiments was verified using actin protein levels. Overall these data confirmed that MCJ regulates c-Jun protein levels (86).

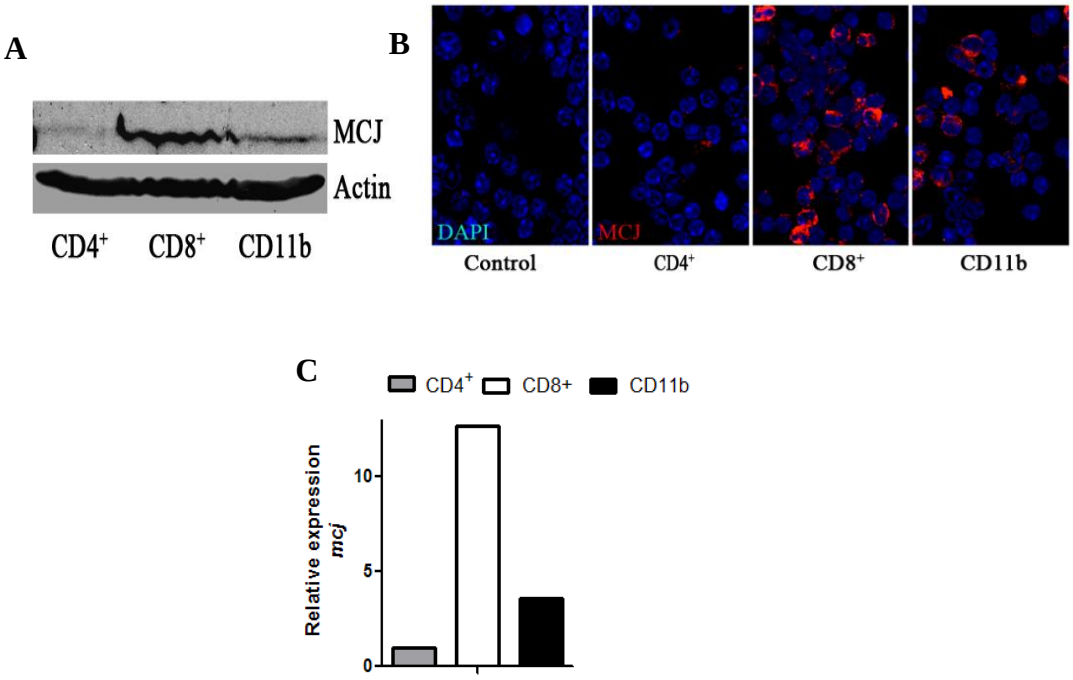

Figure 3.14 MCJ is expressed in macrophages

(A) Immunoblot of purified $CD4^+$ (negative control), $CD8^+$ (positive control) and $CD11b^+$ cells to determine the presence of MCJ protein. (C) qRT-PCR with RNA extracted from$CD4^+$, $CD8^+$ and $CD11b^+$ to determine *mcj* gene expression levels. (B) The cells were fixed, permeabilized and stained with a rabbit anti-mouse MCJ antibody. The nuclei were stained with DAPI.

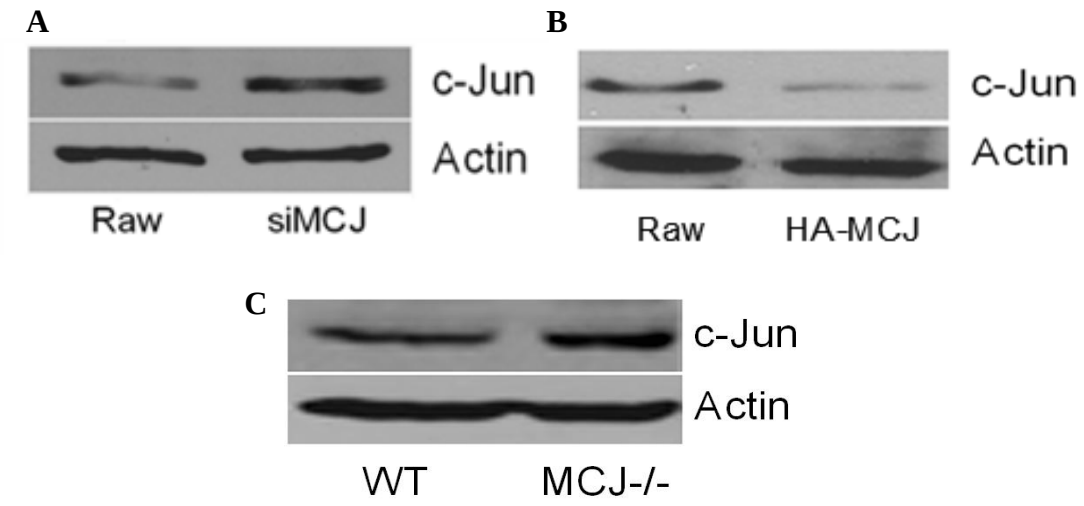

Figure 3.15 MCJ regulates c-Jun protein levels in macrophages

(A) Immunoblot analysis of RAW264.7 and siMCJ cells was performed to determine the c-Jun protein levels. c-Jun levels were also determined by immunoblot analysis in (B) HA-MCJ and(C) MCJ-/- BMDM s. 7% gel was used to run the proteins. Equal loading was measures by determining the levels of Actin protein.

MCJ modulates immune receptor expression and regulates macrophage responses to *B. burgdorferi*

To determine whether MCJ is involved in the regulation of macrophage responses to *B. burgdorferi,* we first analyzed AP-1 transcriptional activation in response to *B. burgdorferi* stimulation using an AP-1-luciferase reporter plasmid. RAW264.7 and siMCJ cells were transfected with an AP-1-LUC plasmid and stimulated with live *B. burgdorferi* (Bb) at 25 MOI for 16 hours, washed and lysed to measure luciferase activity. siMCJ cells showed increase luciferase activity compared to control cells (Fig. 3.16A). We next transfected HA-MCJ cells with the AP-1-luciferase plasmid and assessed luciferase levels in these cells. Over expression of MCJ resulted in reduced levels of AP-1 transcriptional activity compared to control cells (Fig. 3.16B). These results indicate that MCJ regulates c-Jun-mediated responses in macrophages.

To further investigate the role that MCJ plays in macrophage responses to *B. burgdorferi*, we generated BMDM from WT and MCJ-deficient mice and extracted RNA. Since AP-1 regulates the expression of the *tlr1* gene, we then assessed the expression levels of the *tlr1* gene by qPCR. Normalization of gene expression was performed using actin primers. The expression levels of the*tlr1*gene were elevated in MCJ-deficient cells compared to B6 controls (Fig. 3.17A). We next determined the contribution of MCJ to *tlr1*gene expression in siMCJ and RAW264.7 cells. As expected, *tlr1* expression levels were higher in siMCJ cells than controls (Fig. 3.17B). The expression of the *tlr1* gene was also measured in HA-MCJ cells using qRT-PCR. HA-MCJ cells expressed lower levels of *tlr1* compared to control cells (Fig. 3.17C). These results were complemented with the analysis of TLR1 protein levels by Western blot.

MCJ-/- cells had higher levels of TLR-1 thanWT macrophages (Fig. 3.17D). The same result was obtained with siMCJ cells compared to RAW264.7 macrophages (Fig. 3.17E). Consistently, the TLR-1 protein levels in HA-MCJ cells were lower compared to controls (Fig. 3.17F). The results of TLR-1 protein levels in MCJ-/-, siMCJ and HA-MCJ correlated with the *tlr1* gene expression level of these cells. These data show that MCJ regulates TLR-1 expression in macrophages.

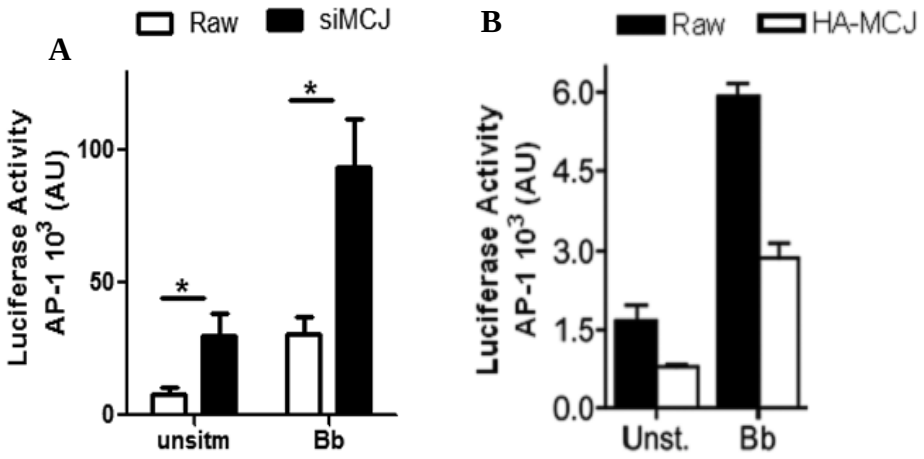

Figure 3.16 MCJ regulates AP-1 activity in responses to *B. burgdorferi*

RAW and siMCJ (A) cells or HA-MCJ cells (B) were transfected with an AP-1-*luc* plasmid and stimulated with live *B. burgdorferi* for 16 hours. Luciferase activity was then measured in cell lysates. The data correspond to the average of triplicate determinations. *, $p < .05$

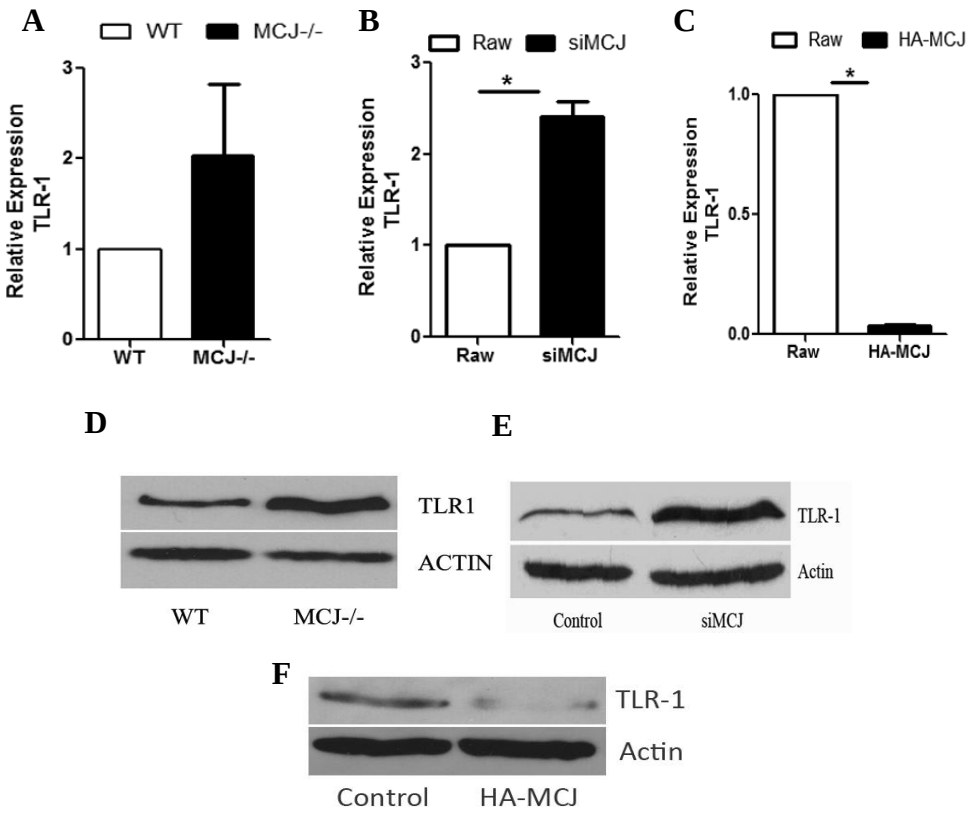

Figure 3.17 *tlr1* gene and protein levels are regulated by MCJ

Real-time RT-PCR for the expression of *tlr1* mRNAs in (A) MCJ-/- BMDM, (B) siMCJ and (C) HA-MCJ cells. Equal input of RNA was assessed by the amplification of the housekeeping gene actin. (D) MCJ-/- BMDM, (E) siMCJ and (F) HA-MCJ cells were also used to determine the levels of TLR1 by immunobloting. Protein input was assessed by immunobloting with an anti-actin Ab. The data correspond to the average of triplicate determinations. *, $p < .05$

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MCJ regulates *tnf* gene expression and its release from macrophages in response to *B. burgdorferi*

TNF is one of the cytokines produced by innate immune cells in response to TLR ligation. We have shown that MCJ is involved in regulating macrophage responses that are c-Jun dependent. To assess whether MCJ has any effect on the production of TNF by macrophages, we first measured *tnf* gene expression in WT and MCJ-deficient BMDM; we also determined *tnf* gene expression in RAW264.7 and siMCJ cells. The cells were incubated for 16 hours with (25 MOI) and without *B. burgdorferi* and RNA was extracted from these cells to generate cDNA. qRT-PCR was performed on each condition using TNF-specific primers. MCJ-deficient macrophages produced increased levels of *tnf* compared to WT controls (Fig. 3.18A). The results from RAW264.7 and siMCJ cells followed the same trend as those obtained with WT and MCJ-/- macrophages showing higher levels of *tnf* gene expression in the presence of repressed MCJ expression (Fig. 3.18 B). In correlation, *tnf* gene expression in HA-MCJ cells was reduced compared to control cells (Fig. 3.18 C).

We next analyzed intracellular levels of TNF. Unstimulated and *B. burgdorferi* stimulated RAW264.7 and siMCJ cells were incubated with Golgi plug and TNF was stained using an anti-TNF conjugated with Allophycocyanin (APC) conjugated antibody. Unstimulated siMCJ cells expressed higher levels of TNF compared to RAW264.7 cells, and TNF levels increased substantially in siMCJ cells stimulated with *B. burgdorferi* compared to stimulated controls (Fig. 3.19 A and B).

Next we measured the levels of secreted TNF. We plated $1x10^6$ cells per well of BMDM that were generated from WT and MCJ-deficient mice. The cells were stimulated

with live *B. burgdorferi* (25 MOI) and the supernatants were collected after 16 hours for TNF determination. Surprisingly, MCJ-deficient macrophages produced significantly reduced levels of TNF compared to WT cells (Fig. 3.20 A). To confirm this finding we performed the same experiment using RAW264.7 and siMCJ cells, showing that TNF was significantly ($P < 0.001$) lower in siMCJ cells compared to controls (Fig. 3.20 B). In correlation, HA-MCJ cells produced substantially higher levels of TNF compared to controls (Fig. 3.20 C). These results showed that TNF secretion is reduced in the absence of MCJ even though the cytokine is produced at higher levels. These findings suggested that MCJ may regulate TNF converting enzyme (TACE) expression and/or activity and therefore, TNF release from the plasma membrane. We first analyzed TACE expression in siMCJ, HA-MCJ and RAW264.7 cells. $1x10^6$ cells were lysed and protein levels were measured by Western blot. The levels of TACE were higher in siMCJ cells and reduced in HA-MCJ cells compared to controls (Fig. 3.21 A). To confirm these findings, RAW264.7 and siMCJ cells were grown in chamber slides, fixed and permeabilized and TACE levels were assessed by fluorescence microscopy. siMCJ cells contained higher levels of TACE compared to control cells (Fig. 3.21 B). cDNA was also generated from $1x10^6$ RAW264.7and siMCJ cells as well as WT and MCJ-/- BMDM to perform qRT-PCR analysis of *tace* gene expression. siMCJ cells expressed higher levels of *tace* (Fig. 3.21 C and D). These results suggested that the level of TACE expression is not a contributing factor in the regulation of TNF protein secretion in cells with reduced MCJ levels. We next determined TACE enzymatic activity in cells with repressed or absent MCJ. $2.5x10^5$ RAW264.7 and siMCJ cells were placed in glass cuvettes with a TNF-FRET substrate peptide and levels of florescence released was measured and recorded, as

indicative of TACE activity. In contrast to higher TACE levels, TACE enzymatic activity was decreased in siMCJ cells compared to controls (Figure 3.22).

It has been demonstrated that Jun proteins control TNF shedding in the epidermis by direct transcriptional activation of tissue inhibitor of metalloproteinase-3 (TIMP-3), an inhibitor of TACE (22). Because MCJ regulates c-Jun stability, we analyzed *timp-3* expression in MCJ-/- BMDM and siMCJ cells by qRT-PCR using specific primers. Unstimulated MCJ-deficient macrophages and siMCJ cells expressed higher levels of *timp-3* compared to controls, with a more substantial increase in siMCJ cells stimulated with *B. burgdorferi* (Fig. 3.23 A and B). To determine whether MCJ-mediated increased TIMP-3 expression plays a role in the regulation of TNF release by MCJ, we transfected RAW264.7 and siMCJ cells with siRNA specific for *timp-3*. The cells were rested for 48 hours, and then stimulated with *B. burgdorferi* for eight hours. TNF levels were measured from control transfected RAW264.7, control transfected siMCJ, and siMCJ cells that were transfected with siTIMP-3. As expected, control transfected siMCJ cell secreted TNF levels that were reduced compared to control transfected RAW264.7 cells (Fig. 3.24 A). In contrast, TNF levels were significantly $P < 0.01$ higher in siTIMP-3 transfected siMCJ cells compared to control transfected siMCJ cells. qPCR was performed using *timp-3* specific primers to ensure that *timp-3* gene expression had been repressed by the siRNA (Fig. 3.24 B). In order to show that the reduced expression of TIMP-3 had resulted in increased TACE activity, we also analyzed fluorescence release from the TACE FRET substrate in siMCJ cells transfected with the*timp-3*-specific siRNA. As expected, siTIMP-3 transfected siMCJ cells had increased TACE activity compared to control transfected siMCJ cells (Figure 3.25), functionally demonstrating that the siRNA

had resulted in repressed TIMP-3 production. Overall, these data demonstrate that MCJ regulates TIMP-3 expression, TACE activity and the release of TNF by macrophages.

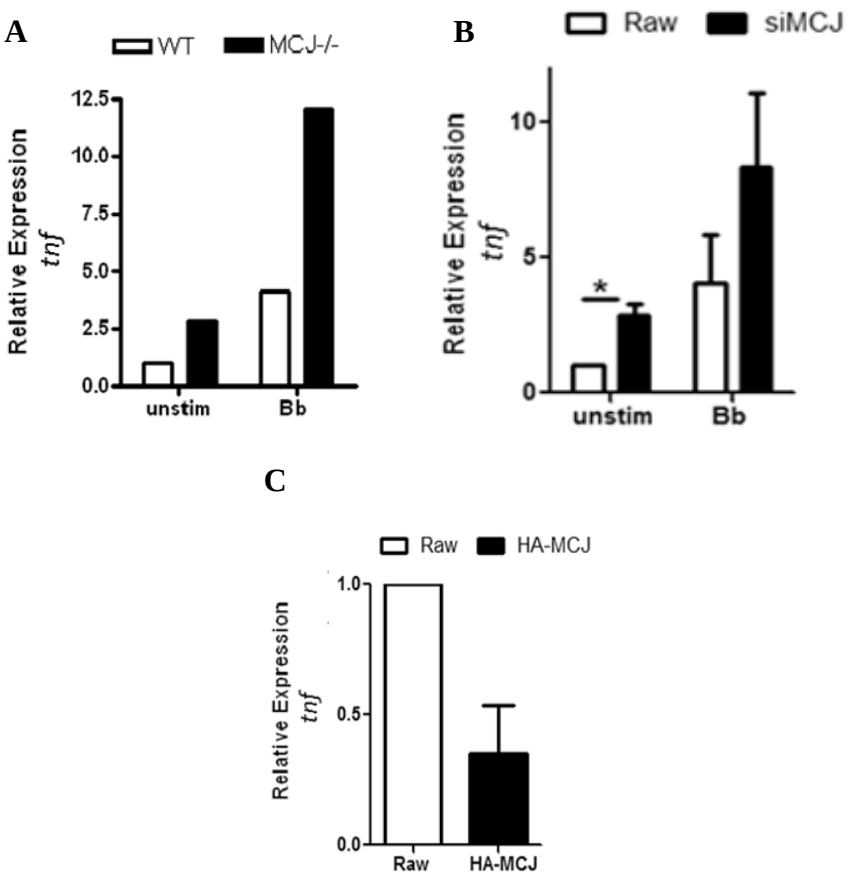

Figure 3.18 MCJ controls *tnf* gene expression in macrophages

(A) Real-time RT-PCR for the expression of *tnf mRNAs* in (A) MCJ-/- BMDM, (B) siMCJ unstimulated or stimulated with *B. burgdorferi* for 16 hours and (C) unstimulated HA-MCJ cells was performed. Equal input of RNA was assessed by the amplification of the housekeeping gene actin. *, $p < .05$

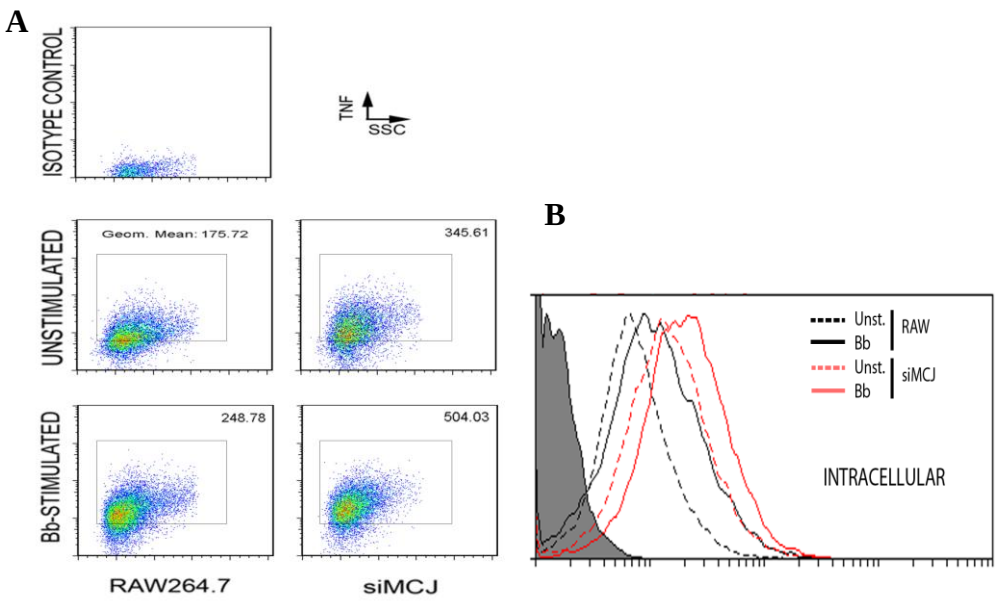

Figure 3.19 Intracellular TNF levels in RAW264.7and siMCJ cells

(A) Intracellular staining to determine TNF levels in RAW264.7 and siMCJ cells Unstimulated and *B. burgdorferi* stimulated for 6 hours in the presence of Golgi plug. (B) Histogram analysis of TNF intracellular staining.

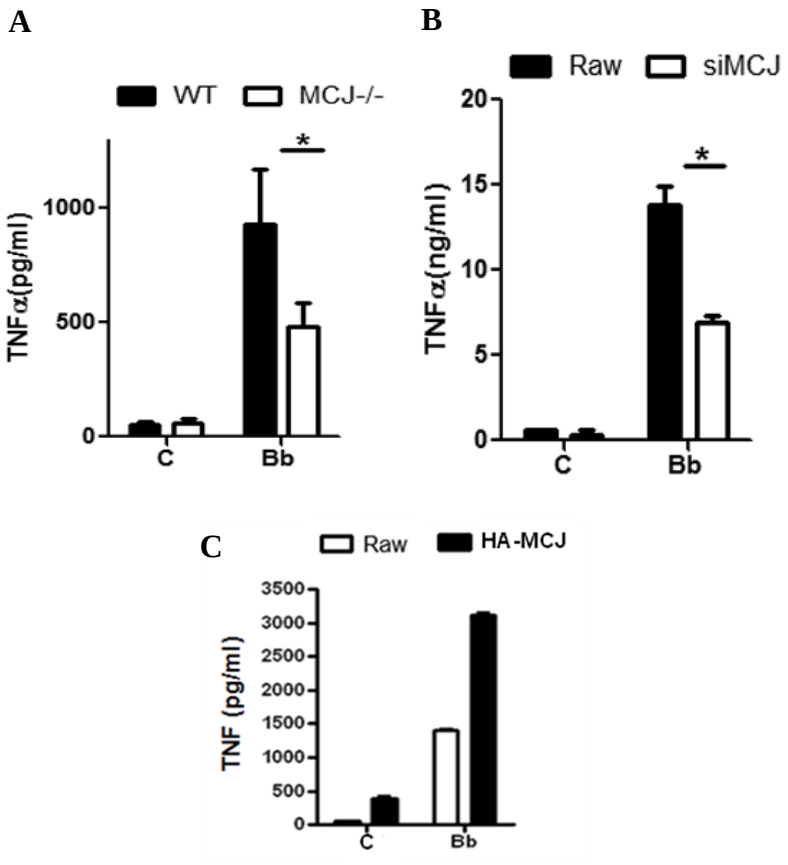

Figure 3.20 MCJ regulates the secretion of TNF in macrophages

TNF levels in the stimulation supernatants from (A) MCJ-/- BMDM, (B) siMCJ and (C) HA-MCJ cells were quantified by ELISA. Cells were stimulated with 25 MOI of *B. burgdorferi* for 16 hours. The results shown are the average plus standard error (SE) *, p< .01

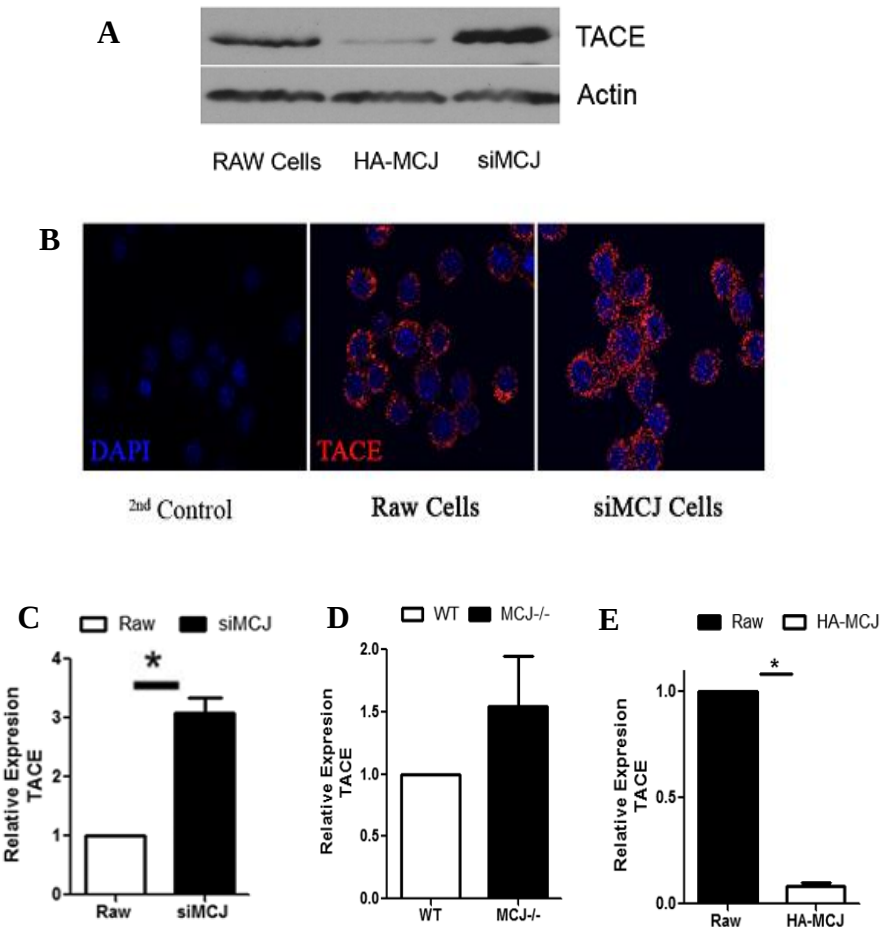

Figure 3.21 Increased TACE levels in cells with repressed or absent MCJ

(A) Immunoblot of TACE protein in RAW, HA-MCJ and siMCJ cells. Protein input was assessed by immunobloting with an anti-actin Ab. (B) Intracellular staining of TACE (PE) levels in RAW and siMCJ cells, and nuclear staining blue (DAPI).(C) Real-time RT-PCR for the expression of *tace* mRNAs in (C) siMCJ (D) MCJ-/- BMDM and (E) HA-MCJ was performed. Equal input of RNA was assessed by the amplification of the housekeeping gene actin. The data correspond to the average of triplicate. *, p< .05

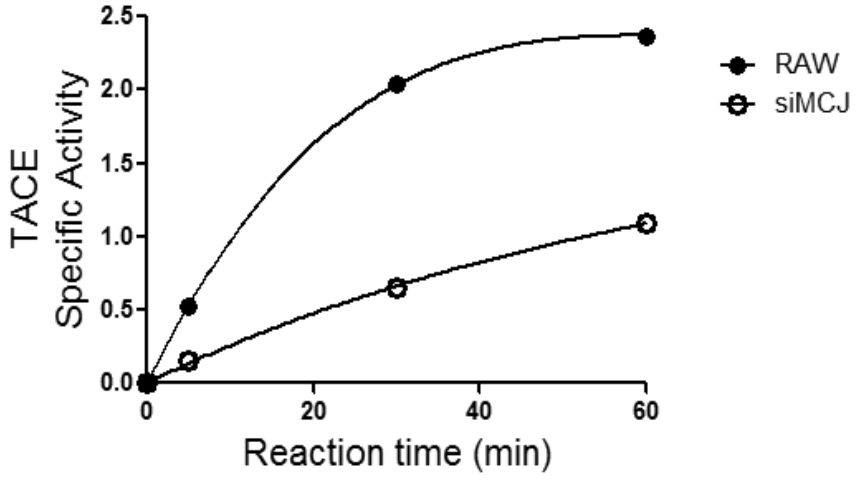

Figure 3.22 Decreased TACE activity in cells with repressed expression of MCJ

Enzymatic activity of TACE was determined in Raw and siMCJ cells using FRET peptide at indicated time points.

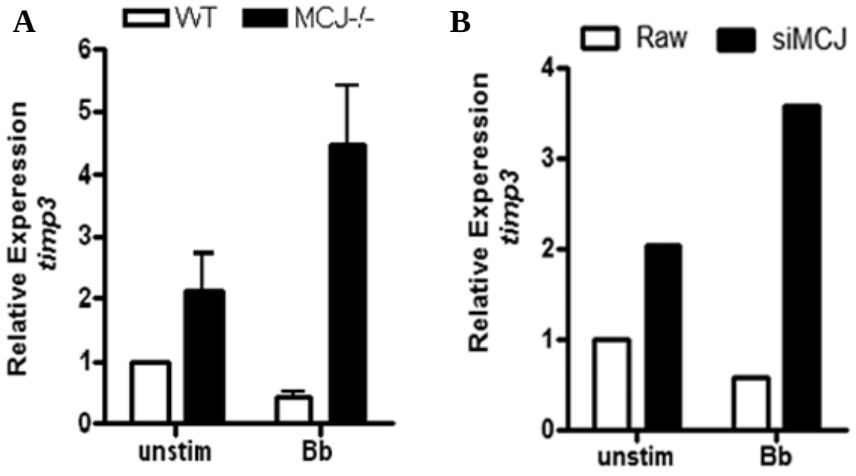

Figure 3.23 MCJ regulates *timp3*gene expression in macrophages

Real-time RT-PCR for the expression of *timp3* mRNAs in (A) MCJ-/- BMDM (B) siMCJ cells unstimulated or *B. burgdorferi* stimulated for 16 hours was performed. Equal input of RNA was assessed by the amplification of the housekeeping gene actin.

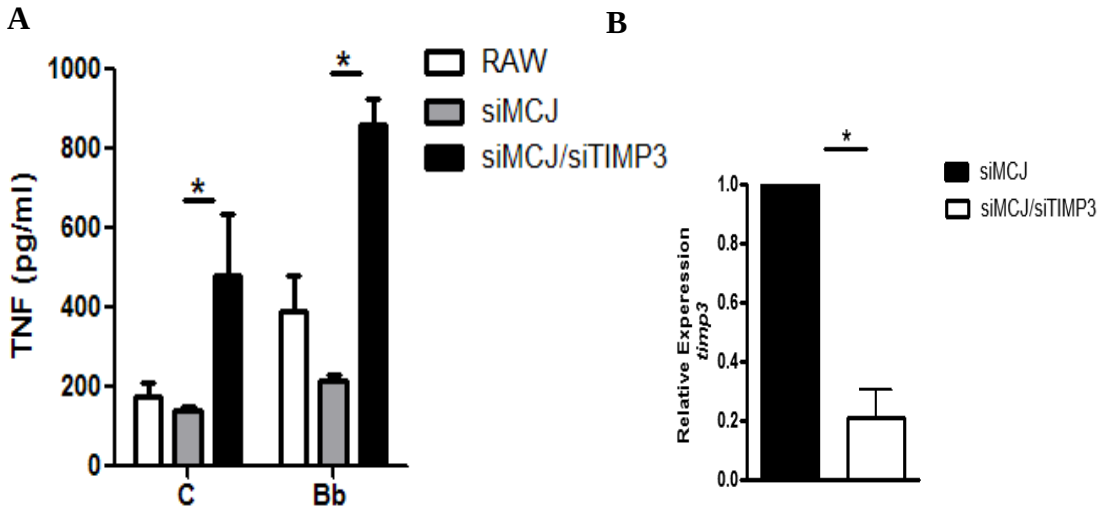

Figure 3.24 Repression of TIMP-3 expression results in increase TNF cytokine secretion in cells with reduced MCJ

(A) RAW264.7 cells were transfected with control siRNA and siMCJ transfected with siTIMP3. TNF levels in unstimulated and stimulation supernatants were quantified by ELISA. Cells were stimulated with 25 MOI of *B. burgdorferi* for 16 hours. (B) qPCR analysis of *timp3* as a control for transfection efficiency. The results shown are the average plus standard error (SE) *, $P < 0.001$ and $p < .01$

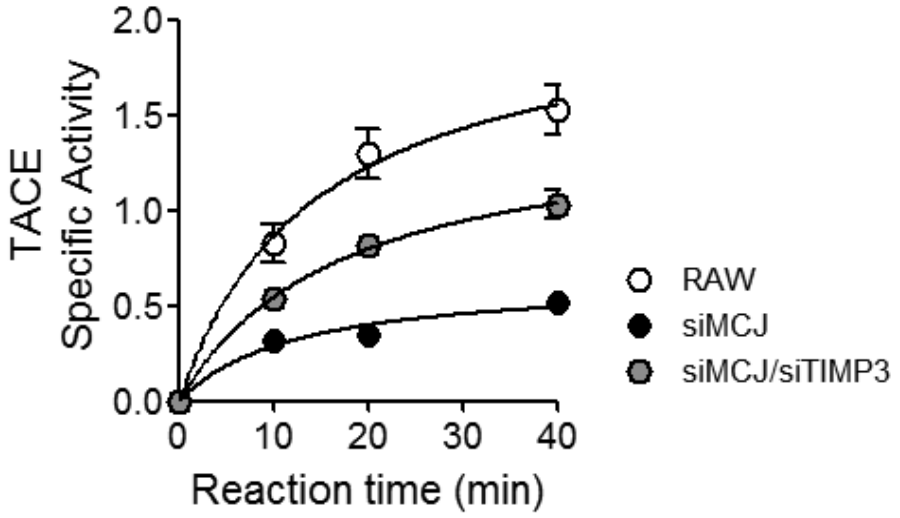

Figure 3.25 Repression of TIMP-3 expression results in increase TACE activity in cells with reduced MCJ

Enzymatic activity of TACE was determined in siMCJ cells transfected with siTIMP3 using FRET peptide at indicated time points. Non specific siRNA transfected RAW264.7 and siMCJ cells were used for control. The data correspond to the average of triplicate determinations.

CHAPTER IV

CONCLUSIONS

The interaction of pathogen-associated molecular patterns with the germ line-encoded TLRs is a fundamental feature of the immune response associated with infection. It is becoming evident that these interactions in innate immune cells have profound consequences for the ability of the body to mount efficient or detrimental responses to pathogens. Thus, deficiencies in TLR1, TLR2, or the adaptor protein MyD88 lead to increased *B. burgdorferi* burdens in mice (37, 102, 103), underscoring the importance of these receptors for the efficient control of infection. Moreover, the severity of pathology associated with infection with *Mycobacterium leprae* is associated with the host's capacity to respond to mycobacterial antigens and is related to TLR expression (104). In addition, TLR ligands are being considered as potential adjuvants that could help boost the response to vaccines compared to the currently limited choices in humans (105, 106). TLR-mediated signals are also required for the successful vaccination of individuals against infectious diseases. For example, the deficient expression of TLR1 in patients that are hyporesponsive to lipidated outer surface protein A of *B. burgdorferi* is associated with decreased responses to the vaccine against the spirochete (37). It is therefore imperative to understand the control of these responses, including how the expression of TLRs is regulated in innate immune cells.

Our results demonstrate that JNK activation upon engagement of TLR1/2 complexes with specific ligands initiates a positive feedback cascade that increases the expression of TLR1. They also show that in our model the ectopic expression of the

receptor restores the capacity of macrophages to respond to *B. burgdorferi* and PAM_3-CSK_4, indicating that in our system, JNK1 activity contributes exclusively to TLR1 expression. Experiments with splenic macrophages derived from deficient mice demonstrated that JNK1, but not JNK2, activity is involved in the control of TLR1 expression, while neither JNK1 nor JNK2 regulates the expression of TLR2 in RAW264.7 cells or primary macrophages. These results correlate with previous observations in the macrophage cell line RAW264.7 that showed no implication of JNK in the expression of the *tlr2* gene (107). Furthermore, our results show that JNK2 also contributes to the induction of TNF in response to *B. burgdorferi* antigens and the TLR1/2 agonist PAM_3-CSK_4 through a mechanism that does not involve the regulation of TLR1. In correlation, the induction of TNF by the TLR4, TLR9, and TLR5 agonists lipopolysaccharide, poly (dI-dC), and flagellin, respectively, was lowered in cells transfected with the plasmid containing dnJNK (Figure1.8D). These data therefore imply non-overlapping functions of the two kinases that have also been demonstrated in T cells and other cell types (108, 109, 110, 111) and that have been associated with differential binding of both isoforms with their substrates (112).

Several reports have shown the regulation of TLR expression by different stimuli, including *B. burgdorferi* stimulation and cytokines (100, 104). Similarly, corticotropin-releasing factor and the urocortins regulate the expression of TLR4 through the activation of the transcription factors PU.1 andAP-1 (113), although the exact mechanisms of regulation have not been described. The regulation of TLR expression may be stimulus dependent, since *B. burgdorferi* does not affect the expression of the *tlr4* gene (data not shown). Similarly, the regulation of *tlr2* gene expression by NF-κB has been documented

(107, 114), and NF-κB binding sites have been described in the *tlr2* promoter (109). However, our results indicate that despite lower NF-κB activation induced by TLR ligands in the absence of JNK activity due to reduced expression levels of TLR1, the levels of TLR2 remained unchanged.

The interaction of *B. burgdorferi* antigens with macrophages potentially occurs through different TLRs: TLR1/TLR2 dimers respond to triacylated lipoproteins, such as lipidated OspA (37), while TLR5 and TLR9 probably contribute to the response of bacterial lysates through their interaction with flagellin and hypomethylated CpG motifs, respectively (116). In turn, these interactions may be physiologically relevant, due to bacterial death in vivo and most importantly, phagocytosis (117). Our results strongly suggest, however, that the predominant response under our experimental conditions occurred through TLR1/TLR2, since (i) the response was mimicked by the use of the TLR1/TLR2 agonist PAM_3-CSK_4 and (ii) the ectopic expression of TLR1 in JNK-repressed RAW264.7 cells completely restored the responses to both *B. burgdorferi* extracts and PAM_3-CSK_4. In light of our results, we propose that the interactions of spirochetal antigens with TLRs result in the activation of JNK. JNK substrates contribute to the up-regulation of the *tlr1* gene, which in turn increases the response of macrophages to TLR1/TLR2 ligands. Thus, we propose that JNK1 contributes to the response of macrophages to *B. burgdorferi* by regulating the expression of TLR1.

Several signaling pathways are activated as a result of the ligation of TLRs with specific ligands. The specific contribution of each pathway to phagocytic responses is still unclear. During infection with *B. burgdorferi*, p38 mitogen-activated protein kinase

controls inflammation (118). Our results indicate that the different signal pathways activated as a result of the interactions between TLRs and their ligands are not redundant, although they may all be needed for a full response to infectious agents. Since macrophage responses to *B. burgdorferi* are largely dependent on TLR-mediated interaction with ligands present in the bacterium, our results can provide the basis for a full understanding of the immune response to this prevalent infectious agent.

Because macrophages are the principal immune cells found during experimental infection with *B. burgdorferi* (119, 120), the interaction between the macrophage and the spirochete has important consequences for the pathology observed during the disease. These interactions result in the activation of complex signaling events that are important for the regulation of macrophage responses to the infectious agent, including the up regulation of surface proteins and the production of inflammatory cytokines. However, many proteins involved in signaling events and the regulation of immune cell activation are unknown or still poorly studied. One such a protein is MCJ. MCJ is a member of the DnaJ protein family of cochaperones. Molecular chaperones are a ubiquitous class of proteins that interact with short stretches of hydrophobic amino acids, typically exposed in partially unfolded proteins. Through such interactions, chaperones function in a broad range of physiological process, facilitating protein folding, and their translocation across membranes as well as the remodeling of multimeric protein complexes (5).

Although a certain amount of interest has been given to the regulation of the *mcj* gene, nothing is known about its biology in innate immune cells and the role it may play in the regulation of immune responses (86). In this study we first determined that MCJ

was expressed in macrophages. We show that MCJ is involved in the regulation of c-Jun in macrophages as has been shown in the MCF7 breast cancer cell line (86). We also Demonstrate that MCJ modulates macrophage responses through the regulation of c-Jun through a complex mechanism. We show that MCJ regulates TLR1 levels in macrophages through the control of c-Jun.

Tumor necrosis factor (TNF) is the main pro-inflammatory cytokine produced and secreted by macrophages (88). Newly synthesized TNF quickly accumulates in the Golgi complex after stimulation and is transported to intermediate compartments before its delivery to the plasma membrane. TNF biological activity is regulated at post-transcriptional levels (89, 90). In this study we have determined the contribution of MCJ to the regulation of TNF production and secretion. We first demonstrated the involvement of MCJ to *tnf* gene expression and showed that in absence of MCJ, *tnf* gene expression substantially increases in both unstimulated and *B. burgdorferi* stimulated cells. We also established the involvement of MCJ in the secretion of TNF from macrophages stimulated with *B. burgdorferi*. Our data show that the level of *tnf* gene expression and its secretion did not correlate, which prompted us to clarify this discrepancy. While intracellular levels of TNF in cells with repressed or absent MCJ was increased, correlating with augmented gene expression, the detection of the cytokine in stimulation supernatants was significantly diminished. TNF is produced as an inactive peptide that is converted to its active and soluble form after cleavage by TACE (91, 92). TACE enzymatic activity is regulated by its inhibitor TIMP-3 (92). This suggested that MCJ could be affecting TACE activity or its inhibitor TIMP-3. In this study we show that TACE activity is reduced in cells lacking MCJ and that this reduction is due to increased

expression of TIMP-3, which is regulated by Jun proteins (94). Because TACE cleaves several other surface proteins, including L-Selectin, TNF Receptor (P75), Notch1 and others (121), our results also suggest that MCJ modulates, through the regulation of TIMP-3 expression, other aspects of cellular function, including cell adhesion and inter-cellular communication. Future work will determine the contribution of MCJ to these and other processes.

In summary, we have demonstrated the contribution of JNK1 and MCJ to the regulation of macrophage responses through the activation and stability of c-Jun, respectively. In turn, the generation of mature TNF is regulated by c-Jun at several levels through the regulation of *tlr1*, *tnf* and *timp-3* gene expression. Therefore, our results place c-Jun as a critical component of the innate immune response to *B. burgdorferi*. Future work will also assess the importance of c-Jun-mediated responses during pathological processes, including infection with *B. burgdorferi*.

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