Pro- and anti-inflammatory mechanisms regulating the mammalian response to *Borrelia burgdorferi*

A dissertation

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ABSTRACT

Borrelia burgdorferi stimulates a strong inflammatory response during infection of a mammalian host. Toll-like receptors (TLRs), especially TLR2/1 heterodimers which recognize bacterial lipopeptides, play the major role in the induction of the inflammatory response to *B. burgdorferi*. Adaptors and co-receptors that mediate this process, as well as the mechanisms by which these adaptors and co-receptors function, are still being discovered. Here we identify integrin $\alpha_3\beta_1$ as a novel regulator for the recognition of bacterial lipopeptides. We demonstrate that the induction of a specific subset of cytokines is dependent upon integrin $\alpha_3\beta_1$-mediated endocytosis of the lipopeptides. In addition, we address an ongoing controversy regarding endosomal recognition of bacterial lipopeptides by demonstrating that TLR2/1 signals from within endosomal compartments as well as from the plasma membrane, and that downstream responses may differ depending upon receptor localization. We propose that the regulation of endosomal TLR2/1 signaling by integrin $\alpha_3\beta_1$ serves as a mechanism for modulating inflammatory responses. This inflammatory response is important for the control and clearance of the infection, but if left unchecked, inflammation damages the host tissue and causes the clinical manifestations of Lyme disease including neuroborreliosis, carditis, or arthritis. To understand the mechanisms of immune regulation employed by the host to control this inflammatory response, we focused additional studies on adrenomedullin, a peptide produced in response to bacterial stimuli that regulates inflammatory responses by modulating the expression of inflammatory cytokines. Specifically, we

investigated the effect of *B. burgdorferi* on the expression of adrenomedullin *in vitro* and *in vivo*, as well as the ability of adrenomedullin to dampen host inflammatory responses to the spirochete. Our results suggest that *B. burgdorferi* increases the production of adrenomedullin, which in turn negatively regulates the *B. burgdorferi*-stimulated inflammatory response. These data identify a novel mechanism by which the host regulates the response to *B. burgdorferi*.

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LIST OF ABBREVIATIONS

AP-1	Activator protein-1
BSK-II	Barbour-Stoenner-Kelley medium
cAMP	Cyclic AMP
CD	Cluster of differentiation
CMA	Control mouse ascites fluid
CPZ	Chlorpromazine
CR3	Complement receptor 3
CRLR	Calcitonin receptor-like receptor
DAB2	Disabled homolog 2
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
GPI	Glycosylphophatidylinsoitol
HEK293	Human embryonic kidney 293 cells
IFN	Interferon
IFN-I	Type I interferon
IKK	Inhibitor of NF-κB kinase
IL	Interleukin
IRAK	IL-1R-associated kinase
IRF	Interferon regulator factors

JAK	Janus kinase
Jnk	c-jun N-terminal kinase
LRR	Leucine-rich repeats
LPS	Lipopolysaccharide
LTA	Lymphotoxin alpha
MAL	MyD88 adaptor-like
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloprotease
MOI	Multiplicity of infection
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa light chain enchancer of activated B cells
NLR	Nod-like receptors
Nod2	Nucleotide binding oligomerization domain-containing 2
Osp	Outer surface protein
OVA	Ovalbumin
Pam_3CSK_4	(*S*)-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser(*S*)-Lys_4-OH trihydrochloride
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cell

PI3-K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKC	Protein kinase C
PPR-γ	Peroxisome proliferator-activated receptor γ
PPR	Pattern recognition receptors
qRT-PCR	Quantitative reverse transcriptase PCR
RAMP	Receptor activity modifying protein
S.D.	Standard deviation
S.E.M.	Standard error of the mean
shRNA	Short hairpin RNA
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TAB	TAK1 binding protein 2
TAK	Transforming growth factor β-activated kinase
TIR	Toll-interleukin 1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein inducing IFN-β
V-ATP-ase	Vacuolar ATP-ase

PRO- AND ANTI-INFLAMMATORY MECHANISMS REGULATING THE MAMMALIAN RESPONSE TO *BORRELIA BURGDORFERI*

CHAPTER 1

INTRODUCTION

Lyme disease

Lyme disease is the most common vector-borne disease in the United States, with approximately 30,000 confirmed cases reported and an additional 10,000 probable cases predicted in 2009 (Center for Disease Control). In the early 1980s, *Borrelia burgdorferi* was identified as the causative agent of this disease (20, 27, 195).

B. burgdorferi is introduced into the human host through the bite of an infected *Ixodes scapularis* tick. Since *B. burgdorferi* does not produce any known toxins or proteases (48, 121), the symptoms of Lyme disease are thought to be primarily the result of an over-exuberant and poorly controlled inflammatory response. At the site where the bacteria enter the host, the localized immune response results in a characteristic skin rash known as erythema migrans. At this early stage of disease, patients may also experience nonspecific symptoms such as fevers and chills, fatigue, headache, and muscle and joint aches. If the infection remains untreated, the bacteria disseminate and colonize distal tissues such as the nervous system, heart, and large, weight-bearing joints (Figure 1.1) (173). At these sites, the interaction between *B. burgdorferi* and the host results in the induction of an inflammatory response. The production of chemokines and cytokines is important for the recruitment of immune cells to the site of infection, and for the differentiation and activation of these cells. *B. burgdorferi* also induces the production of matrix metalloproteases (MMPs), which are zinc- and calcium-dependent proteases important in the degradation and remodeling of

mammalian extracellular matrix (ECM) (152). These MMPs are thought to contribute to the tissue damage observed in Lyme patients and, together with the localized inflammatory environment, to the clinical manifestations of Lyme disease including neuroborreliosis in the nervous system, carditis in the heart, or arthritis in the large, weight-bearing joints (173). Indeed, several groups have demonstrated a role for MMPs in models of arthritis (37, 74, 83).

Figure 1.1

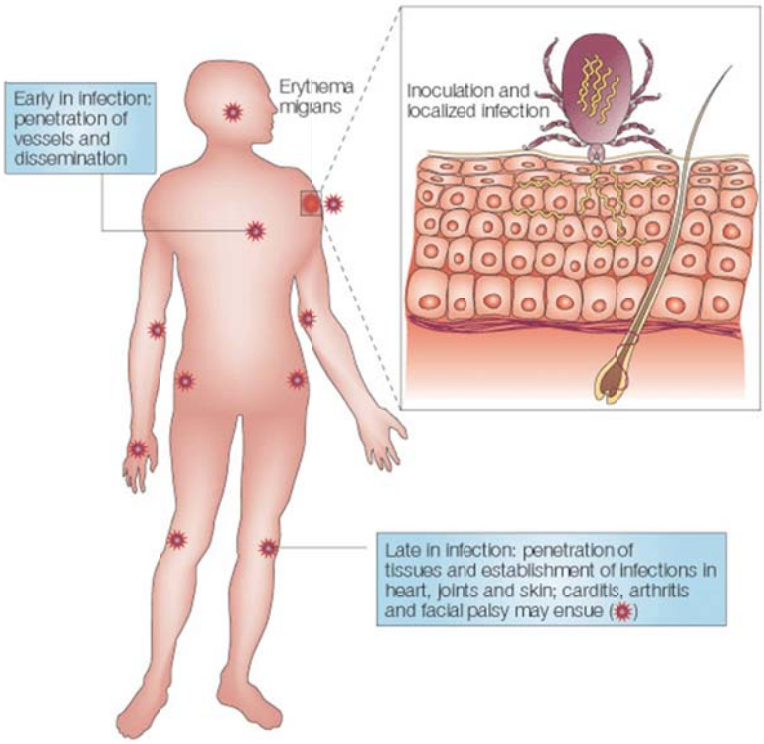


Figure 1.1 *B. burgdorferi* disseminates from the point of entry and stimulates inflammatory symptoms (from Rosa et al. 2005. *Nat Rev Microbiol*. 3: 129-143. Reprinted with permission.)

B. burgdorferi is introduced into the human host through the bite of an infected *Ixodes scapularis* tick. If the infection is left untreated, the bacteria disseminate to distal tissues such as the nervous system, heart, and large, weight-bearing joints. An over-exuberant inflammatory response causes the clinical manifestations of Lyme disease including neuroborreliosis, carditis, and arthritis.

Borrelia burgdorferi

B. burgdorferi is a species of bacteria belonging to the class *Spirochaetes*. Species within this class are characterized by their unique helical morphology (Figure 1.2A). The membrane structure of spirochetes consists of an inner membrane and an outer membrane which surround periplasmic flagella and a flexible cell wall (13, 93, 106, 120) (Figure 1.2B and C). The periplasmic flagella are anchored at either end of the bacterial cell, cross in the center of the cell, and rotate in opposite directions (13, 69, 129, 150). The position and properties of the flagella confer to *B. burgdorferi* both its helical shape as well as its corkscrew-like pattern of motility (150, 176). This motility allows the bacteria to penetrate mammalian tissues to colonize the host and establish infection (69, 118).

The infectious cycle of *B. burgdorferi*

The enzootic life cycle of *B. burgdorferi* is maintained through infections of tick and vertebrate hosts (Figure 1.3). Uninfected *Ixodes scapularis* larvae acquire the bacteria when feeding on an infected mammal, such as the white-footed mouse, *Peromyscus leucopus.* After molting, the nymphs, and later the adults, transmit the infection to uninfected mammals (including humans) during their next blood meal (95).

The segmented genome of *B. burgdorferi*

The genetic composition of *B. burgdorferi* is unique compared to that of other bacterial species in that it contains more plasmids than any other known bacterial

species. This highly segmented genome is composed of a small, linear chromosome of approximately 900 kb and 21 linear and circular plasmids which total approximately 650 kb (60, 197) (Figure 1.4). While the advantage for maintaining 22 separate replicons remains unknown, the strict maintenance of these plasmids throughout the natural infection cycle of *B. burgdorferi* (28, 29, 33, 164, 169, 190, 196, 207, 220, 231) suggests that the genes contained on these plasmids are important to the survival and transmission of *B. burgdorferi* between its various hosts.

One important class of genes encoded on the plasmids of *B. burgdorferi* encodes the lipoproteins. The genome of *B. burgdorferi* encodes more than 160 lipoproteins, and expression of these lipoproteins is altered throughout the infection cycle in response to the different environments the bacteria encounter in each host (158). The paradigmatic example of the regulation of lipoproteins during transmission from one host to the next is the regulation of outer surface protein A (OspA) and OspC expression, *B. burgdorferi* expresses OspA, which is important for colonization of the tick midgut (163). As the tick feeds on a mammalian host, the spirochetes begin to proliferate (50, 178) and down-regulate OspA. Simultaneously, the bacteria up-regulate the expression of OspC (178). As the spirochetes pass through the tick salivary glands and into the mammalian host, OspC interacts with Salp15, a tick salivary protein (172) which protects the bacteria from the mammalian immune response by a variety of mechanisms which include the inhibition of $CD4^+$ T cell activation, the inhibition

B. burgdorferi-induced cytokine expression by dendritic cells, and the protection of *B. burgdorferi* from serum-mediated killing (8, 94, 108, 109, 172, 177).

Figure 1.2

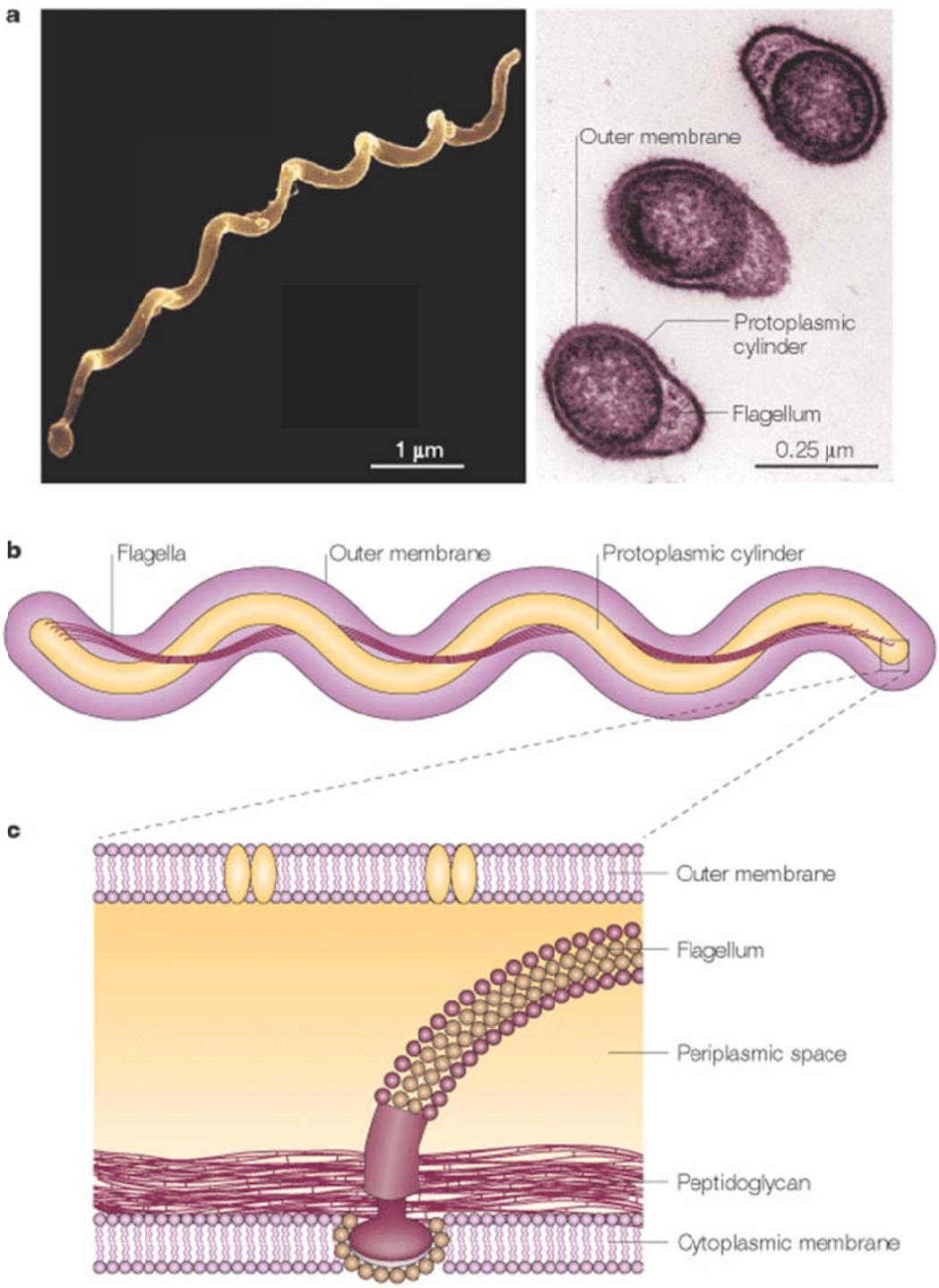

a
1 µm
Outer membrane
Protoplasmic cylinder
Flagellum
0.25 µm
b
Flagella
Outer membrane
Protoplasmic cylinder
c
Outer membrane
Flagellum
Periplasmic space
Peptidoglycan
Cytoplasmic membrane

Figure 1.2 The morphology of *B. burgdorferi* (from Rosa et al. 2005. *Nat Rev Microbiol*. 3: 129-143. Reprinted with permission.)

(A) Scanning (left) and transmission (right) electron microscopy images of *B. burgdorferi*. **(B and C)** Diagram of spirochetes. Flagella are inserted in the inner membrane near the two ends of the bacterial cells. Flagella extend the length of the bacterial cell, wrapping around the bacterial cells within the periplasmic space.

Figure 1.3

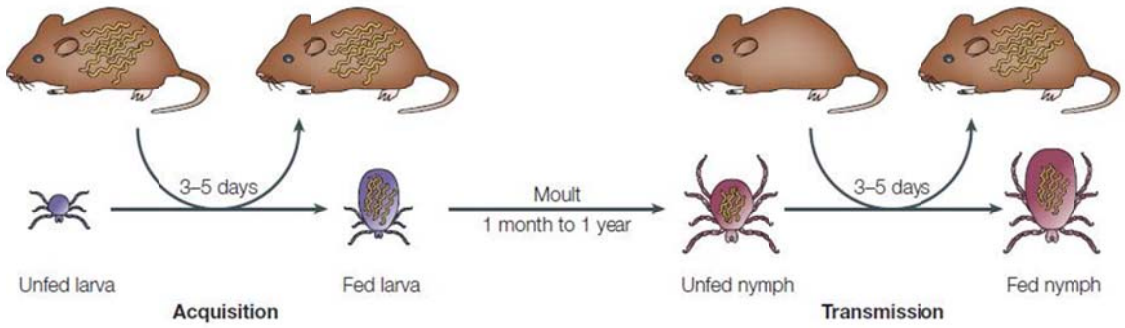


Figure 1.3 The infectious cycle of *B. burgdorferi* (from Rosa, et al. 2005. *Nat Rev Microbiol*. 3: 129-143. Reprinted with permission.)

B. burgdorferi is maintained in an infectious cycle between tick and vertebrate hosts. Uninfected *Ixodes scapularis* ticks acquire the infection while feeding on infected mammals, and transmit the infection to uninfected mammals during subsequent blood meals.

Figure 1.4

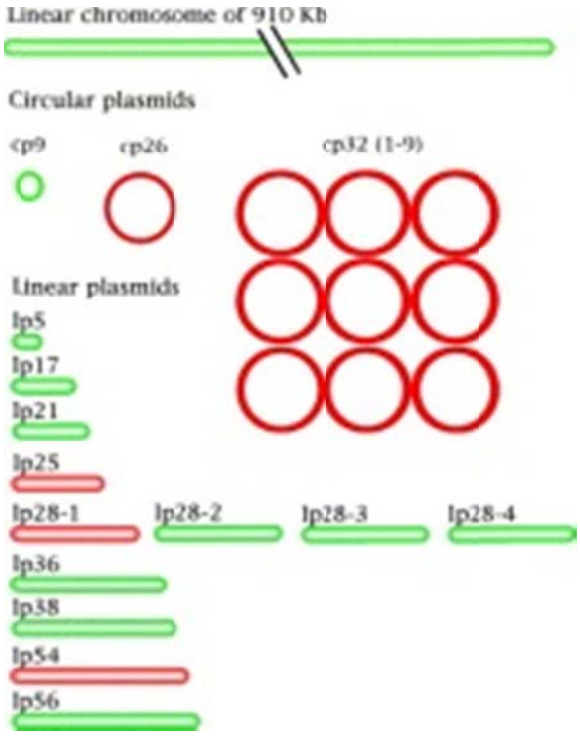


Figure 1.4 The segmented genome of *B. burgdorferi* (from Stewart et al. 2004. *Plasmid*. 53: 1-13. Reprinted with permission.)

Plasmids shown in red are required for infectivity and persistence in the tick and vertebrate hosts. Linear plasmids (lp), circular plasmids (cp).

Innate immune recognition of *B. burgdorferi*: Toll-like receptors

The symptoms of Lyme disease are thought to be caused primarily by an over-exuberant and poorly controlled inflammatory response. Thus, understanding the mechanisms that induce this inflammatory response is of great significance. The immune response to *B. burgdorferi* is mediated by the recognition of spirochetes or their products by pattern recognition receptors (PRRs) of the innate immune system which recognize pathogen-associated molecular patterns (PAMPs). Among the PRRs, toll-like receptors (TLRs), especially TLR2 (86, 188, 189, 215, 216), are thought to play the major role in mediating inflammatory responses to *B. burgdorferi* (16, 22, 134, 188).

The TLR family contains 10 human receptors and 12 murine receptors that recognize a diverse array of PAMPs and activate downstream inflammatory cascades (204). TLRs are type I transmembrane proteins that contain three domains: a ligand-binding extracellular domain composed of leucine-rich repeats (LRRs), a transmembrane domain, and an intracellular Toll-interleukin 1 receptor (TIR) domain which recruits the necessary adaptor proteins for signal transduction (204).

Despite similarity in structure, each TLR induces the expression of a different subset of inflammatory mediators. This specificity of response is achieved through the recognition of different ligands, the cooperation with different co-

receptors, the localization to different subcellular compartments, and the recruitment of different signaling adaptor proteins.

TLRs recognize a variety of PAMPs

Although the extracellular domain of each TLR is composed of LRRs, each TLR recognizes a different microbial pattern (Figure 1.5). TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 recognize primarily lipoproteins, lipids, and protein PAMPs, whereas TLR3, TLR7, TLR8, and TLR9 recognize nucleic acid PAMPs. The precise molecular interactions that allow for such diversity in ligand recognition are not fully understood, but the crystallization of some receptor-ligand structures suggests that different TLRs utilize different ligand-binding surfaces (24, 105, 116, 133).

TLRs and co-receptors

Early models of TLR-ligand interactions proposed simple, direct interactions between a TLR and a ligand without the aid of other molecules. It is now understood that other adaptor molecules and co-receptors mediate and alter these interactions, resulting in greater diversity in the response elicited by ligands and pathogens. Multiple receptors and adaptor molecules that influence TLR signaling have now been identified. These include cluster of differentiation 14 (CD14) (179, 225), Nod-like receptors (NLRs) (122), dectin-1 (56, 186), scavenger receptors such as macrophage receptor with collagenous structure (MARCO) (23) and scavenger receptor A (SR-A) (6, 219), and integrins (59, 65,

98, 110, 166). For the majority of these receptors, the mechanism by which they influence TLR signaling is not well defined.

Cellular localization of TLRs

The cellular localization of TLRs also conveys specificity to the inflammatory response (Figure 1.6) (14). It is traditionally thought that the TLRs which recognize lipoproteins, lipids, and protein PAMPs primarily function at the plasma membrane, while the nucleic acid-sensing TLRs recognize ligands in and signal from intracellular compartments such as endosomes and lysosomes. Localization is important, and each TLR is localized to and signals from the cellular compartment where it is most likely to encounter ligand. Protein and lipid PAMPs are typically expressed on the surface of pathogens and readily ligate plasma membrane TLRs, whereas nucleic acids are inaccessible to TLR recognition until the pathogen has been internalized and degraded in lysosomal compartments. Although TLR2 has been found in endosomal compartments (211), TLR4 was, until recently, considered unique among the TLRs because TLR4 transduces signals both from the plasma membrane and from endosomal compartments (96, 111).

Figure 1.5

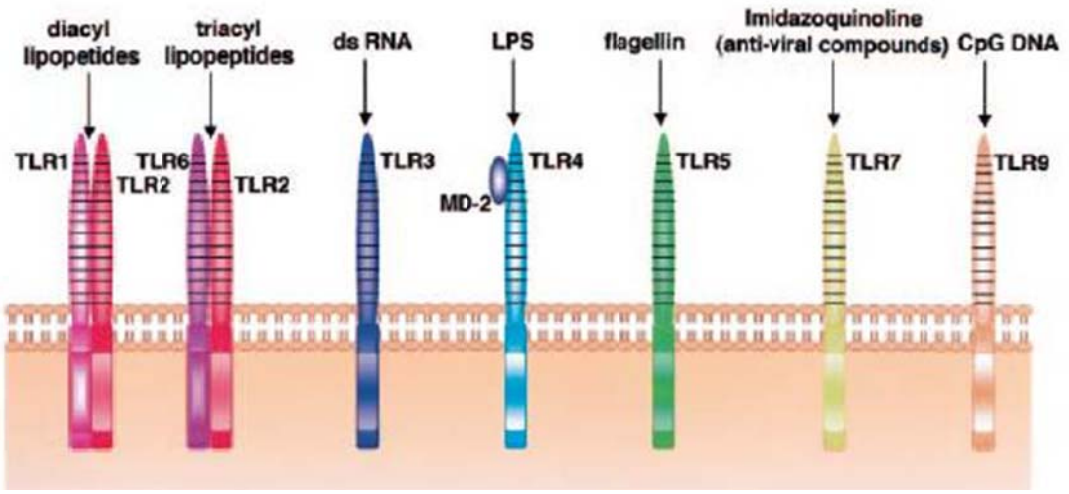


Figure 1.5 TLRs recognize a variety of PAMPs (from Akira et al. 2003. *J Biol Chem*. 278: 38105-38018. Reprinted with permission.)

TLR1, TLR2, TLR5, TLR6, and TLR4 recognize lipoproteins, lipids, and protein PAMPs. TLR7, TLR8, and TLR9 recognize nucleic acid PAMPs. Toll-like receptor (TLR), Double stranded (ds), lipopolysaccharide (LPS).

Figure 1.6

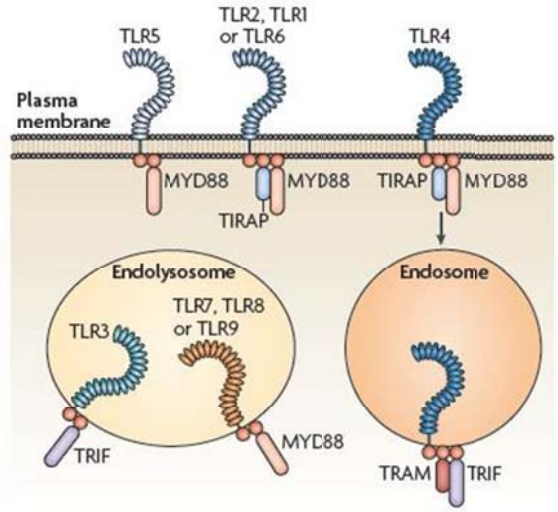


Figure 1.6 Cellular localization of TLRs (from Barton et al. 2009. *Nat Rev Immunol*. 9: 535-542. Reprinted with permission.)

TLR1, TLR2, TLR5, and TLR6 transduce signals from the plasma membrane. TLR3, TLR7, TLR8, and TLR9 transduce signals from intracellular compartments such as endosomes and lysosomes. TLR4 transduces signals both from the plasma membrane and from endosomal compartments.

TLR adaptor proteins

Transduction of intracellular signals downstream of TLRs requires the recruitment of TIR domain-containing adaptor proteins to the cytoplasmic TIR domains of TLRs. These adaptor proteins are differentially recruited to the TLRs, and activate distinct signaling pathways, thus contributing to the specificity of response downstream of each TLR (Figure 1.7) (159).

Myeloid differentiation factor 88 (MyD88) was the first TLR adaptor identified (135), and is utilized by all TLRs except TLR3. The recruitment of MyD88 to TLR2 or TLR4 at the plasma membrane requires an intermediate adaptor protein known as MyD88 adaptor-like (MAL, or TIRAP) (57, 91, 92, 110, 221). MAL contains a phosphatidylinositol 4,5-biphosphate (PIP_2)-binding domain, which allows for its recruitment into PIP_2-rich microdomains in the plasma membrane where TLR2 and TLR4 are localized (110). MAL then recruits MyD88 to facilitate signaling. MyD88-dependent signaling (with or without MAL) from the plasma membrane ultimately results in the activation of nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and the induction of pro-inflammatory cytokines.

MyD88 is recruited (independent of MAL) to the TIR domains of endosomal TLR7, TLR8, and TLR9 to transduce signals (159). At this subcellular localization, recruitment of MyD88 results not only in the activation of NF-κB and

the induction of inflammatory cytokines, but also in the activation of interferon regulator factors (IRFs) and the subsequent induction of type 1 interferon (IFN-I).

TLR3 is the only TLR that does not signal in a MyD88-dependent manner. Rather, TLR3 recruits TIR-domain-containing adaptor protein inducing IFN-β (TRIF) (162). The signaling pathways activated downstream of this adaptor ultimately result in the activation of IRF3 and the induction of IFN-β, as well as in the activation of NF-κB and the induction of inflammatory cytokines.

TLR4 utilizes all known TIR domain containing adaptor proteins, depending upon its localization. From the plasma membrane, TLR4 recruits MAL and MyD88 to activate NF-κB and induce inflammatory cytokines (57, 110, 221). From endosomes, TLR4 recruits TRIF-related adaptor molecule (TRAM), which in turn recruits TRIF to activate both NF-κB-dependent induction of inflammatory cytokines, as well as IRF-dependent induction of IFN-β (111).

Figure 1.7

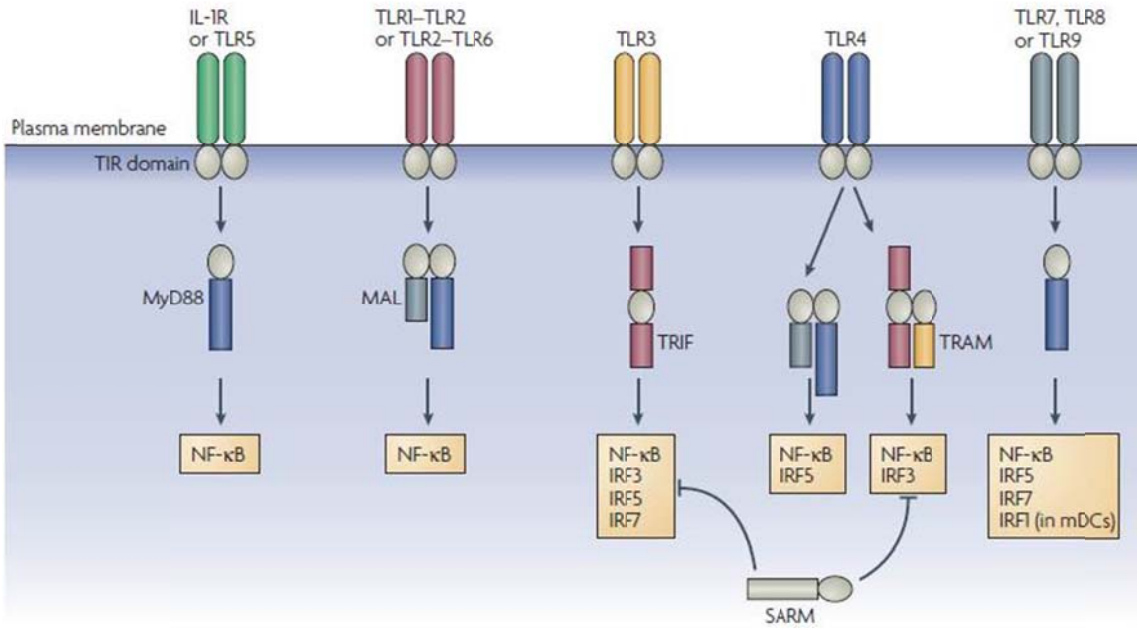


Figure 1.7 TLR adaptor proteins (from O'Neill et al. 2007. *Nat Rev Immunol*. 7: 353-364. Reprinted with permission.)

The TIR domain-containing adaptor proteins are differentially recruited to TLR receptors, thus regulating the activation of different transcription factors.

The TLR response to *B. burgdorferi*

Several TLR family members recognize components of *B. burgdorferi*. TLR2 recognizes *B. burgdorferi* lipoproteins (86, 188, 215, 216), TLR5 recognizes *B. burgdorferi* flagella (188), and TLR7, TLR8, and TLR9 recognize *B. burgdorferi* nucleic acids (148, 168).

The ligation of TLRs by *B. burgdorferi* results in the initiation of inflammatory signaling pathways. Our lab, as well as others, has identified many of the signaling molecules activated in response to *B. burgdorferi* infection. These include novel protein kinase C (PKC) isoforms (PKC δ, PKC ε, PKC η, and PKC θ (187)), mitogen-activated protein kinases (MAPKs) (p38 and c-jun N-terminal kinase (JNK) (7, 19)), and Janus kinase/signal transducer and activator of transcription (JAK/STAT) proteins (STAT 3 and STAT 6 (7, 19)).

As with other TLR ligands, the signaling pathways activated in response to *B. burgdorferi* result in the production of inflammatory mediators. The production of chemokines and cytokines is important for the recruitment of immune cells to the site of infection, and for the differentiation and activation of these cells. TLR signaling in response to *B. burgdorferi* also induces the production of MMPs. Since *B. burgdorferi* does not produce any known toxins or proteases, these inflammatory mediators are thought to be responsible for the tissue damage observed in Lyme patients.

TLR2 elicits the strongest response to *B. burgdorferi*

Of the TLRs known to recognize *B. burgdorferi* ligands, TLR2 elicits the strongest response to *B. burgdorferi* (86, 188, 215, 216). The borrelial components which activate TLR2 signaling are the lipoproteins expressed by *B. burgdorferi*, many of which contain the (*S*)-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser(*S*)-Lys_4-OH trihydrochloride (Pam_3CSK_4) modification (86). Such tri-acylated lipid modifications are recognized by TLR2/1 heterodimers.

According to current paradigm, TLR2/1 recognizes *B. burgdorferi* lipoproteins at the plasma membrane and recruits MAL and MyD88 to the receptor complex. MyD88 then recruits IL-1R-associated kinase (IRAK) family members. The IRAK kinases phosphorylate and activate tumor necrosis-factor (TNF) receptor-associated factor 6 (TRAF6), which in turn forms a complex with the E2 ubiquitination complex Ubc13 and Uev1A. This complex synthesizes lysine 63-linked polyubiquitin chains to TRAF6 to activate and recruit transforming growth factor β-activated kinase 1 (TAK1) and TAK1-binding protein 2 (TAB2). This complex phosphorylates kinases upstream of p38 and JNK MAPKs, and the inhibitor of NF-κB kinase (IKK) complex, leading to NF-κB activation and translocation into the nucleus and the production of inflammatory cytokines. However, some evidence suggests that TLR2 is found within endosomes (211). Whether TLR2 transduces signals from these compartments is an area of controversy. Original models suggested that TLR2 can only signal from the plasma membrane because one of its signaling adaptors, MAL, is not recruited to

endosomal compartments (110, 111). However, Barbalat et al. recently showed that TLR2 signals from endosomal compartments in response to viral, but not bacterial, ligands (11). Later, Dietrich et al. demonstrated that bacterial lipopeptides induce TLR2 signaling from endosomal compartments (51). Each of these studies suggested that TLR2 signals from endosomes to induce IFN-I, but not inflammatory cytokines (11, 51).

In Chapter 2 of this dissertation, we demonstrate that TLR2/1-mediated recognition of bacterial lipopeptides occurs not only at the plasma membrane, but also from within sub-cellular compartments. TLR2/1 signaling from endosomal compartments results in the induction of a different subset of inflammatory mediators than signaling from the plasma membrane, and this subset includes both IFN-I and inflammatory cytokines. Our data provide support for the emerging concept that localization and context of TLR recognition of ligands alters the inflammatory response.

TLR2 associates with co-receptors to facilitate the recognition of ligands. For instance, TLR2 heterodimerizes with TLR1 to recognize tri-acylated lipopeptides and with TLR6 to recognize diacylated lipopeptides. The ligand-binding capabilities of TLR1 and TLR6 allow TLR2 to distinguish between these two types of lipopeptides. The hydrophobic domain of TLR2 binds only two lipid chains, and thus requires the hydrophobic domain of TLR1 to accommodate the third lipid chain of tri-acylated lipopeptides (24, 105). Conversely, TLR6 does not

contain the same hydrophobic domain as in TLR1, so TLR2/6 dimers cannot bind the same ligands as TLR2/1 dimers (24, 105). Other co-receptors for TLR2 complexes have recently been identified. For instance, CD36 and dectin-1 both cooperate with TLR2/6 heterodimers to mediate the recognition of and, in some cases phagocytosis of, ligands (156, 183, 198, 199).

Another co-receptor for TLR2 is CD14 (23, 79, 156, 209). CD14 is a glycosylphophatidylinsoitol (GPI)-linked protein with an extracellular domain that, like those of TLRs, is composed of LRR and binds lipid and lipoprotein PAMPs. The mechanism by which CD14 cooperates in TLR2/1-mediated responses is unknown. In Chapter 3 of this dissertation, we demonstrate that CD14 participates in the endocytosis of Pam_3CSK_4, thus facilitating recognition of Pam_3CSK_4 by endosomal TLR2/1. These data suggest a novel mechanism of cooperation between TLR2/1 and CD14 in the recognition of triacylated lipopeptides.

Innate immune recognition of *B. burgdorferi*: Phagocytosis

As discussed above, TLR7 and TLR9, which are localized to sub-cellular compartments, transduce inflammatory signals in response to *B. burgdorferi* (148, 168). Phagocytosis and lysosomal processing of the organism are likely required prior to recognition by these sub-cellular TLRs. Indeed, previous work from our laboratory and others suggests that phagocytosis of *B. burgdorferi* is important in the activation of inflammatory signals in macrophages and monocytes (49, 188).

The mechanisms regulating phagocytosis of *B. burgdorferi* are not entirely understood. MyD88-dependent signals are important for the efficient phagocytosis of the spirochetes by macrophages *in vitro* (188, 189), and MyD88-deficient mice exhibit increased bacterial burden *in vivo* (16, 134). However, whether signaling through individual TLRs is sufficient to induce phagocytosis of *B. burgdorferi*, or whether this process requires cooperation of several TLRs remains unclear. Deficiency in single TLRs (including TLR2) does not affect the efficiency of phagocytosis *in vitro* (188). However, TLR2-deficient mice, like MyD88-deficient mice, exhibit increased bacterial burden *in vivo* (216), suggesting that TLR2 signaling is important for the control of bacterial burden. Further work is necessary to elucidate the role of TLR2 and other TLRs in the control of bacterial burden *in vivo.*

In addition to TLRs, other receptors have been hypothesized to participate in the phagocytosis of *B. burgdorferi*. For instance, some groups have proposed a role for complement receptor 3 (CR3) in the first step in phagocytosis, the attachment of *B. burgdorferi* to host cells (41). However, there is precedent that internalization of particles can involve a series of receptors that separately mediate attachment and internalization (88, 192, 232). This suggests that the receptors which tether *B. burgdorferi* to the host cell do not necessarily also trigger the phagocytosis of *B. burgdorferi*. Thus, the receptors and mechanisms regulating the phagocytosis of *B. burgdorferi* remain largely unknown.

Since both TLR2/1 signaling (86, 188, 215, 216) and phagocytosis (49, 188) are important for the inflammatory response to *B. burgdorferi*, it has been hypothesized that TLR2/1 may recognize *B. burgdorferi* ligands from subcellular compartments (188). However, as discussed above, while TLR2 has been shown to localize to the endosome, whether TLR2 initiates inflammatory signaling from these compartments remains controversial. Therefore, prior to our studies, whether TLR2/1 recognizes *B. burgdorferi* ligands in and transduces signals from endosomal compartments remained unknown.

Innate Immune recognition of *B. burgdorferi*: Integrins

Integrins are adhesion receptors which are expressed on the plasma membrane and mediate interactions between a cell and its environment. These obligate heterodimers are formed by the noncovalent association of two type I membrane glycoproteins: an α-subunit (180 kDa) and a β-subunit (95 kDa) (9). The association of a particular α-subunit with a particular β-subunit results in the formation of a unique ligand-binding site and confers ligand specificity to the heterodimer (Figure 1.8).

Integrins exist in equilibrium between an inactive (“closed”) conformation with low affinity for ligand, and an active (“open”) conformation with high affinity for ligand. Inactive integrins must be activated to reveal the ligand-binding site, recognize its ligand, and transmit the necessary signals to allow the cell to respond to its environment.

Figure 1.8

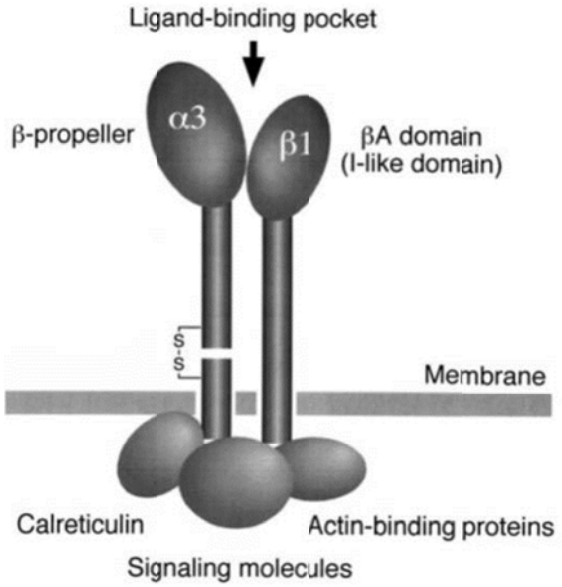


Figure 1.8 Integrin structure (from Tsuji. 2004. *J Membrane Biol*. 200: 115-132. Reprinted with permission.)

Integrin heterodimers are formed by the noncovalent association of two type I transmembrane subunits. The extracellular ligand-binding pocket of the integrin is formed from the association of a particular α-subunit with a particular β-subunit. The cytoplasmic domains recruit signaling proteins to initiate signal transduction cascades.

Integrins as adhesion receptors

Integrins mediate many physiological functions in a variety of cell types, many of which are related to their role as adhesion receptors. Endogenous integrin ligands include ECM proteins such as fibronectin, collagen, and laminin (97), as well as receptors and surface proteins on neighboring cells. Interactions between integrins and these endogenous ligands facilitate cell-ECM and cell-cell interactions which are necessary for cellular adhesion and motility, and tissue development and differentiation (97).

Integrins as endocytic receptors

Integrin function is regulated, in part, by the recycling and redistribution of integrins throughout the plasma membrane. This process is achieved through the internalization of integrins and subsequent trafficking through recycling endosomes (34, 35, 107, 165). The endocytosis of integrins occurs through either clathrin-dependent or clathrin-independent mechanisms. In clathrin-dependent endocytosis, a NXXY motif in the cytoplasmic tail of the β-subunit interacts with clathrin adaptor proteins such as AP2 or disabled homolog 2 (DAB2) to recruit the integrin to clathrin-coated structures (36). In clathrin-independent endocytosis, integrins localize to caveolae where endocytosis requires caveolin 1 (62, 184). Integrin-mediated endocytosis also regulates the function of other receptors such as epidermal growth factor receptor (EGFR) and tyrosine kinase receptors, as well as the recycling of ECM components during tissue remodeling and development (36).

Integrins as signaling receptors

In addition to their roles in adhesion and endocytosis, integrins also transduce bi-directional signals across the plasma membrane (97, 180). In one direction, signaling cascades originate from within the cell to activate integrins and increase their affinity for ligand (136). This “inside-out” signaling is achieved when external stimuli trigger the activation of the Ras-family small GTP-binding protein Rap1 (52, 54, 145) and the recruitment of the cytoskeletal protein talin to the NPX[Y/F] motif in the cytoplasmic domain of β-subunit of inactive integrins (200). The recruitment of talin induces conformational changes and the activation of the integrin (Figure 1.9).

Once an integrin is activated, “outside-in” signaling is initiated in response to ligand binding. The cytoplasmic domains of integrins are not themselves enzymatically active, so outside-in signaling is dependent upon the recruitment of signaling and adaptor proteins to the cytoplasmic domains of the integrins (227). The clustering of ligand-associated, active integrins increases the recruitment of signaling molecules, and amplifies the signaling cascades (30) (Figure 1.9).

The signaling pathways activated downstream of integrin ligation are complex, and signaling downstream of a particular integrin often crosses with signaling from other integrins or other cellular receptors. The consequences of these various signaling pathways include changes in gene transcription, cell

proliferation, actin rearrangements, cell migration, and anoikis (tissue detachment/apoptosis).

Figure 1.9

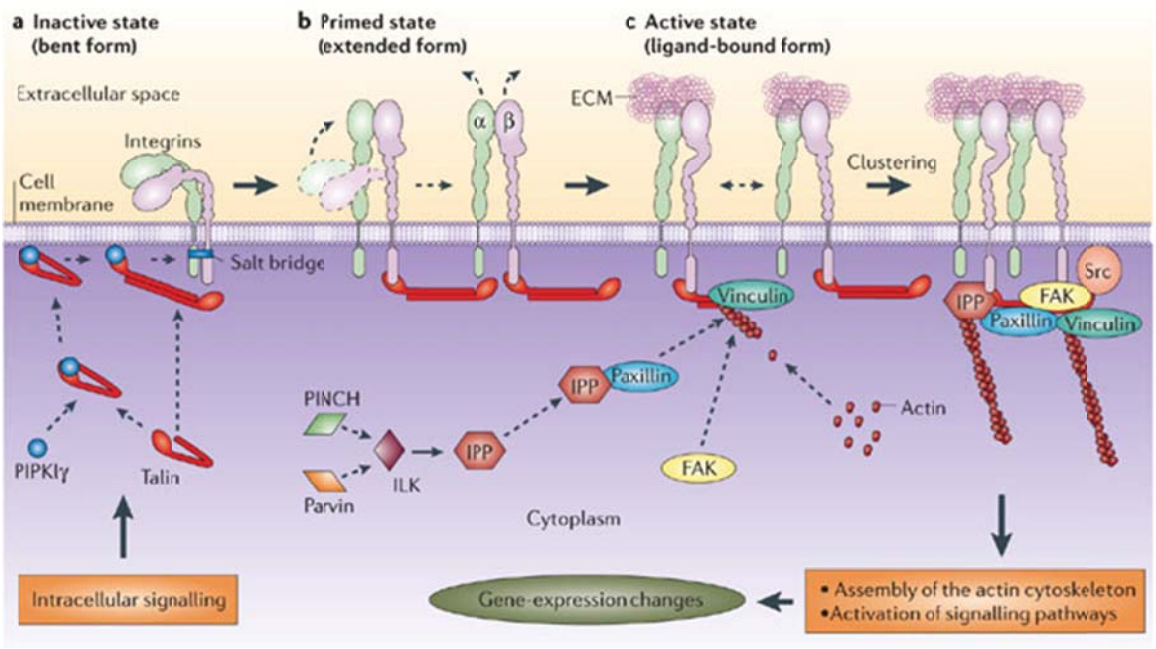


Figure 1.9 Bidirectional integrin signaling (from Legate et al. 2006. *Nat Rev Mol Cell Biol*. 7: 20-31. Reprinted with permission.)

(A and B) Inactive integrins are activated through the recruitment of talin to the cytoplasmic tail of the β-subunit. **(C)** The activated integrins reveal their ligand-binding site and interact with their ligands. These ligated integrins cluster with other ligated integrins and recruit structural and signaling proteins to mediate cellular responses to the extracellular environment.

The role of integrins in pathogen recognition

In addition to the functions of integrins in normal physiology, integrins also mediate interactions between pathogens and the host. Adherence to host tissue is a necessary first step of pathogen colonization, and many bacterial and viral pathogens have evolved to interact with host adhesions receptors, including integrins, to achieve this adherence (181). Some pathogens, such as *Yersinia spp., Shigella spp*., and *Escherichia coli,* express proteins that interact directly with mammalian integrins. Alternatively, bacteria such as *Staphylococcus aureus* and *Bartonella spp.* express proteins which interact with host ECM protein, and thus interact indirectly with the host integrins which recognize these ECM proteins. Such interactions are thought to facilitate the attachment to and colonization of host tissue.

Similar to their role in endocytosis of host proteins, integrins also mediate internalization of pathogens by host cells. After binding to β_1 integrins, bacterial pathogens such as enteropathogenic *Yersinia* species (100) and *Staphylococcus aureus* (53, 191) are endocytosed by the host cells. In addition, viruses such as human cytomegalovirus (212) and Kaposi's sarcoma-associated herpes virus (4, 63) invade target cells via interactions with integrin $\alpha_v\beta_3$.

The role of integrins in the response to *B. burgdorferi*

B. burgdorferi binds to several integrins on mammalian cells (42). *B. burgdorferi* attaches to activated human platelet cells through an interaction with integrin

$\alpha_{IIb}\beta_3$ (45). It has been proposed that this interaction with activated platelets, which in turn attach to the walls of blood vessels, may allow the spirochetes to colonize cardiac tissue in spite of the high force of the blood flowing through the vessel (45). Expression of integrin $\alpha_{IIb}\beta_3$ is limited to platelets, but *B. burgdorferi* also interacts with integrins more ubiquitously expressed in the mammalian host such as integrins $\alpha_V\beta_3$ and $\alpha_5\beta_1$ (46). These interactions are also thought to contribute to the ability of *B. burgdorferi* to colonize the various tissues where these integrins are expressed.

The means by which *B. burgdorferi* interacts with these integrins has not been completely elucidated. *B. burgdorferi* expresses a number of ECM-binding proteins which interact with fibronectin (73, 171), decorin (75, 76), and collagen (229). These ECM proteins, once bound to the bacterial surface, could mediate indirect interactions between *B. burgdorferi* and integrins.

In addition, an integrin-binding protein has been identified in the *B. burgdorferi* proteome. This protein, known as P66, is expressed on the surface of spirochetes, and is therefore accessible to integrin binding during infection of the human host (26, 170). Recombinant P66 binds to both integrin $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ (43, 44), suggesting that this protein may facilitate *B. burgdorferi* attachment to these integrins. Whether P66-integrin interactions are necessary for the colonization of the host is unknown.

The role of integrin $\alpha_3\beta_1$ in the response to *B. burgdorferi*

As discussed above, integrins do not only mediate adhesion, but also transduce signals across the cell membrane. Indeed, integrin signaling triggers inflammatory responses through activation of focal adhesion kinase (FAK), MAPKs, activator protein-1 (AP-1), and NF-κB (68). However, whether integrins regulate inflammatory signaling cascades in response to *B. burgdorferi* was not addressed in the previous studies. Thus, our laboratory sought to determine whether integrins participate in the inflammatory response to *B. burgdorferi*.

Previous work from our laboratory examined the role of integrins in the inflammatory response to *B. burgdorferi* in human chondrocyte cells. This cell type was chosen due to the hypothesis that these cells interact with *B. burgdorferi* in the joints and produce inflammatory chemokines to recruit lymphocytes to the joint. In this experimental system, chondrocytes were pre-treated with function-inhibiting antibodies targeting several α integrin chains prior to infection with *B. burgdorferi*. Of the antibodies tested, only the antibodies targeting integrin $\alpha_3\beta_1$ inhibited the transcription of MMPs induced by *B. burgdorferi* (18) (Figure 1.10). These data suggested that integrin $\alpha_3\beta_1$ regulates the inflammatory response to *B. burgdorferi*. To confirm these findings, the expression of integrin $\alpha_3\beta_1$ was reduced in human chondrocytes using siRNA. Indeed, cells transfected with integrin α_3-targeting siRNA exhibited a significant decrease in the induction of inflammatory cytokines and chemoknes in response

to *B. burgdorferi,* as compared with cells transfected with control siRNA (18) (Figure 1.11).

Taken together, these results demonstrated for the first time a role for an integrin in regulating the inflammatory response to *B. burgdorferi*. However, the mechanism by which integrin $\alpha_3\beta_1$ regulates the inflammatory response to *B. burgdorferi* was not defined.

Figure 1.10

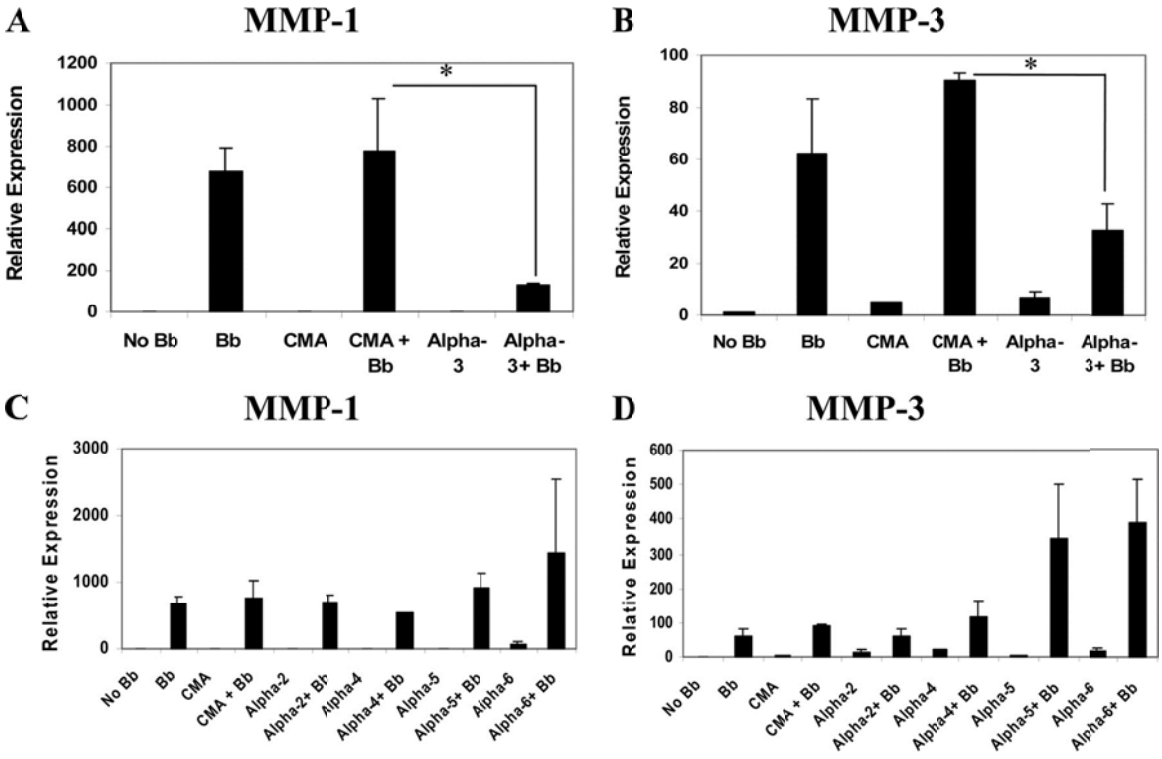


Figure 1.10 Integrin $\alpha_3\beta_1$ regulates *B. burgdorferi*-induced MMP expression (from Behera et al. 2006. *J Immunol*. 177: 657-664. Reprinted with permission.)

Primary human chondrocyte cells were pre-treated with monoclonal anti-integrin $\alpha_3\beta_1$ **(A and B)** or monoclonal antibodies targeting integrin α_2, integrin α_4, integrin α_5, or integrin α_6 **(C and D)** prior to infection with *B. burgdorferi* at multiplicity of infection (MOI) 10 for 24 hours. Expression of MMP-1 **(A and C)** or MMP-3 **(B and D)** was measured by qRT-PCR. Shown are the combined data from four **(A and B)** or two **(C and D)** independent experiments showing mean MMP expression relative to uninfected cells (arbitrarily set to 1, error bars represent S.D.). * $p < 0.05$. Control mouse ascites fluid (CMA), matrix metalloprotease (MMP).

Figure 1.11

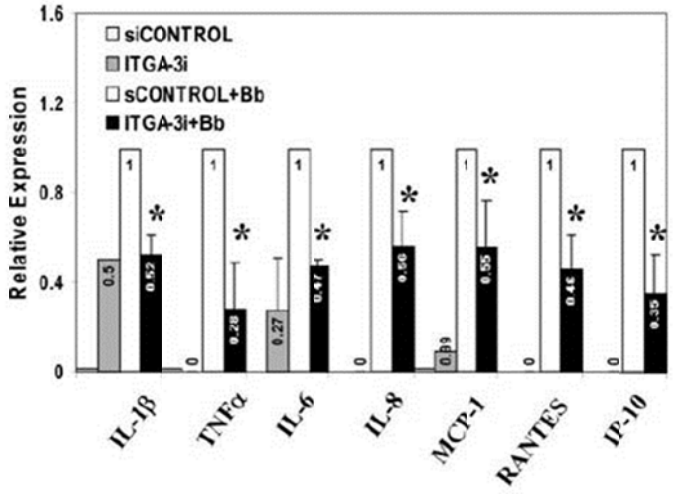


Figure 1.11 Integrin $\alpha_3\beta_1$ regulates *B. burgdorferi*-induced expression of pro-inflammatory cytokines and chemokines (from Behera et al. 2006. *J Immunol*. 177: 657-664. Reprinted with permission.)

Primary human chondrocyte cells were transfected with siRNA targeting integrin α_3 (ITGA3i) or control siRNA (siCONTROL) prior to infection with *B. burgdorferi* (Bb) at MOI 10 for 24 hours. Expression of inflammatory cytokines and chemokines was measured by qRT-PCR. Shown are the combined data from four independent experiments showing mean cytokine or chemokine expression relative to uninfected cells (arbitrarily set to 1, error bars represent S.D.). * $p < 0.05$.

We hypothesized that integrin $\alpha_3\beta_1$ may itself transduce inflammatory signaling cascades that ultimately lead to the induction of inflammatory cytokines and chemokines. To test this hypothesis, we examined integrin $\alpha_3\beta_1$-dependent responses in human embryonic kidney (HEK293) cells, which express integrin $\alpha_3\beta_1$, but do not express the TLRs known to elicit responses to *B. burgdorferi*. HEK293 cells do not produce inflammatory cytokines (such as IL-8) in response to *B. burgdorferi* (Figure 1.12A). However, HEK293 cells stably transfected with a TLR2-epxressing plasmid do produce IL-8 (Figure 1.12A) in an integrin $\alpha_3\beta_1$-dependent manner (Figure 1.12B). These data suggest that integrin $\alpha_3\beta_1$ may not mediate inflammatory responses to *B. burgdorferi* in the absence of TLRs.

Figure 1.12

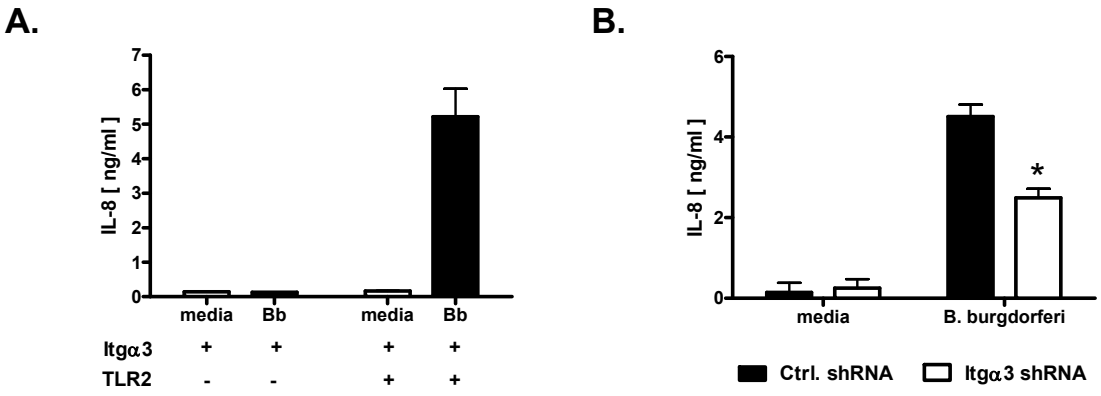


Figure 1.12 Integrin $\alpha_3\beta_1$ does not mediate inflammatory responses without TLRs

(A) HEK293 cells stably transfected with a TLR2-expressing plasmid or a control plasmid were stimulated with *B. burgdorferi* (Bb) MOI 10 for 6 hours. IL-8 secretion was measured by ELISA. Shown are the data from one representative experiment showing mean IL-8 secretion (error bars represent S.D. of triplicates). **(B)** HEK293 cells expressing TLR2 were stably transduced with integrin α_3-specific shRNA (Itgα3 shRNA) or non-targeting shRNA (Ctrl. shRNA) and stimulated with *B. burgdorferi* MOI 10 for 6 hours. IL-8 secretion was measured by ELISA. Shown are the data from one representative experiment showing mean IL-8 secretion (error bars represent S.D. of triplicates). * p = 0.05. Integrin $\alpha_3\beta_1$ (Itgα3), Toll-like receptor 2 (LTR2).

Integrins as co-receptors for TLRs

An additional role for integrins in host defense is through cooperation with other innate immune receptors such as TLRs. Several groups have demonstrated a necessary role for integrin $\alpha_M\beta_2$ in the induction of an inflammatory cytokine response to the TLR4 ligand lipopolysaccharide (LPS) (98, 110, 166). Specifically, integrin $\alpha_M\beta_2$ regulates the generation of PIP_2 in the plasma membrane, which is necessary for the recruitment of MAL to the cytoplasmic tail of TLR4 to initiate signaling. In addition, a recent publication demonstrated a role for integrin $\alpha_v\beta_3$ in the regulation of TLR2/1-mediated responses to a number of stimuli including Pam_3CSK_4 (65). This cooperation was suggested to be mediated through the interaction of Pam_3CSK_4 with vitronectin, the ECM ligand for integrin $\alpha_v\beta_3$. It was proposed that integrin $\alpha_v\beta_3$ mediates the attachment of Pam_3CSK_4 to macrophages, leading to the clustering of the lipopeptide with TLR2/1 at the cell surface and facilitating signaling.

In Chapter 2 of this dissertation, we demonstrate that human macrophage inflammatory responses to the TLR2/1 ligand Pam_3CSK_4 require integrin $\alpha_3\beta_1$. However, the mechanism by which integrin $\alpha_3\beta_1$ regulates TLR2/1 function is not through attachment and clustering of ligand at the cell surface as proposed for integrin $\alpha_v\beta_3$ (65), but rather through the endocytosis of Pam_3CSK_4. Our data provide a new mechanism for the cooperation of integrin and TLR receptors.

Mechanisms that control *B. burgdorferi*-induced inflammation

The inflammatory response to *B. burgdorferi* is necessary for the control of infection and clearance of the spirochetes. However, if left unchecked, inflammation damages host tissue and causes the clinical manifestations of Lyme disease. To avoid an over-exuberant and potentially damaging inflammatory response, host cells employ multiple mechanisms for controlling inflammation.

B. burgdorferi induces the production of anti-inflammatory cytokines

In addition to the pro-inflammatory response induced by *B. burgdorferi* described above, the recognition of *B. burgdorferi* by PRRs also induces the production of anti-inflammatory cytokines both *in vitro* and *in vivo*. Indeed, when co-cultured with *B. burgdorferi*, peripheral blood mononuclear cells (PBMCs) from both humans and rhesus monkeys, as well as murine bone marrow-derived macrophages, increase the expression of interleukin (IL) 10 (25, 66, 67, 67, 125, 175) in a TLR2- and CD14-dependent manner (66). IL-10 then modulates the *B. burgdorferi*-induced inflammatory response by decreasing the production of pro-inflammatory cytokines *in vitro* (25, 125, 151) and by regulating inflammatory manifestations of disease *in vivo* (25, 126). However, IL-10 is not sufficient to control *B. burgdorferi*-induced inflammation.

Chronic infection with *B. burgdorferi* results in tolerance

Another potential mechanism by which the host regulates the *B. burgdorferi*-induced inflammatory response is through the development of tolerance during chronic infection. The induction of tolerance during chronic infections is thought to be as important for host survival as is the control of the bacterial burden. Tolerance dampens the potentially damaging inflammatory response and protects the host from inflammatory symptoms. Mechanisms of tolerance have recently been explored in experimental models of chronic *B. burgdorferi* infection.

The hypothesized role for tolerance arose as a result of studies involving mice deficient in PRRs. Rather than the expected decrease in inflammatory symptoms, mice lacking TLR2, MyD88, CD14, or nucleotide binding oligomerization domain-containing 2 (Nod2), exhibit increased inflammatory symptoms during infection with *B. burgdorferi* (16, 22, 134, 167, 174, 216). The increased inflammatory phenotypes observed in these studies were originally explained by the increased bacterial burden in these mice (16, 22, 134, 216). However, studies of $Nod2^{-/-}$ and $CD14^{-/-}$ macrophages co-cultured with *B. burgdorferi* have suggested an important role for these receptors in mediating tolerance to the spirochetes.

Several studies have suggested a role for Nod2 in the induction of tolerance under experimental conditions which mimic chronic infection (80, 81). Indeed, although Nod2 mediates pro-inflammatory responses to *B. burgdorferi in vitro*

(39, 161, 167), recent studies of chronic infection both *in vitro* and *in vivo* suggest that Nod2 is also important for the development of tolerance to prolonged stimulation either through Nod2 itself or through other PRRs (167). Furthermore, TLR signaling is negatively regulated through the activation of suppressor of cytokine signaling (SOCS) proteins. However, in $CD14^{-/-}$ macrophages, these signaling pathways are altered, leading to decreased SOCS activity and to increased inflammatory signaling through TLRs (174). These data suggest that, in addition to its role in the early induction of inflammation, CD14 also participates in the later regulation of TLR signaling in response to *B. burgdorferi*. Additional studies will be required to determine whether deficiency in TLR2 or MyD88 also influence mechanisms of tolerance.

Adrenomedullin

Adrenomedullin is a mammalian protein that negatively regulates inflammatory processes. It is expressed by numerous cells as a preproadrenomedullin peptide, which is further processed to yield two mature and active peptides, proadrenomedullin N-terminal 20 peptide and the 52-amino acid adrenomedullin (85). Adrenomedullin is constitutively secreted from cells in its immature, inactive form (103, 202), and is activated after secretion by amidation of its C-terminus (119).

Induction of adrenomedullin production

Adrenomedullin is produced by cells such as lymphocytes and macrophages (123, 124, 154, 228) in response to several bacterial pathogens (5, 113, 124, 223, 228) and by inflammatory environments (87, 90, 123, 124, 210).

Adrenomedullin signaling in target cells

Mature and active adrenomedullin transmits signals through a G protein-coupled receptor known as calcitonin receptor-like receptor (CRLR) which requires interaction with a receptor activity modifying protein (RAMP) for transport to the plasma membrane (144). The signal transduction pathways downstream of this receptor are not well defined, but the ligation of CRLR by adrenomedullin results in an elevation in the level of the secondary messenger cyclic AMP (cAMP) within the cell, as well as an increase in intracellular calcium concentrations (101,

185). The interaction of adrenomedullin with its receptor and the subsequent signaling events ultimately results in tissue-specific changes in gene expression.

Adrenomedullin modulates inflammatory responses

Adrenomedullin possesses immunomodulatory activities which have been demonstrated to down-regulate inflammatory processes in a variety of different models both *in vitro* (102, 112, 147, 218) and *in vivo* (32, 70-72, 160, 205, 223, 224). The immunomodulatory effects of adrenomedullin involve the suppression of a T_H1 pro-inflammatory response and the induction of anti-inflammatory cytokines and increase in the regulatory T cell population (70).

In particular, adrenomedullin modulates the inflammatory symptoms observed in experimental models of arthritis. The administration of exogenous adrenomedullin protects mice from arthritis in the collagen-induced murine model of rheumatoid arthritis (70). In addition, adrenomedullin has been detected in the synovial fluid of rheumatoid arthritis patients at concentrations significantly higher than that in the synovial fluid of the less inflammatory osteoarthritis patients (40, 142, 155). A significant portion of the adrenomedullin peptides detected in these synovial fluid samples are active, as demonstrated by the inhibition of IL-6 secretion from the patients' synoviocytes *in vitro* (155).

Many groups have demonstrated that adrenomedullin causes the down-regulation of inflammatory cytokines in a target cell (102, 112, 147, 218).

However, each of these experimental models involved the activation of cells through a single receptor (i.e., LPS stimulating the production of inflammatory cytokines through TLR4 (218)). Little is known about the ability of adrenomedullin to down-regulate an inflammatory response induced by a whole bacterium that activates cells through numerous receptors.

In Chapter 4 of this dissertation, we demonstrate that adrenomedullin expression is increased by macrophages co-cultured with *B. burgdorferi* as well as in the synovial fluid of Lyme disease patients. Furthermore, the addition of exogenous adrenomedullin to *B. burgdorferi*-stimulated macrophages decreases the induction of pro-inflammatory cytokines. Our data suggest that adrenomedullin regulates the host response to *B. burgdorferi*, a complex organism that stimulates cells through multiple receptors.

CHAPTER 2

HUMAN INTEGRIN $\alpha_3\beta_1$ REGULATES TLR2 RECOGNITION OF LIPOPEPTIDES FROM ENDOSOMAL COMPARTMENTS

Note: The data in this chapter has been published.

Marre ML, Petnicki-Ocwieja T, DeFrancesco AS, Darcy CT, Hu LT. 2010. Human integrin $\alpha_3\beta_1$ regulates TLR2 recognition of lipopeptides from endosomal compartments. *PLoS One*. 5(9): e12871.

CHAPTER 2 ABSTRACT

TLR2/TLR1 heterodimers recognize bacterial lipopeptides and initiate the production of inflammatory mediators. Adaptors and co-receptors that mediate this process, as well as the mechanisms by which these adaptors and co-receptors function, are still being discovered. Using shRNA, blocking antibodies, and fluorescent microscopy, we show that macrophage responses to the synthetic TLR2/1 ligand Pam_3CSK_4 are dependent upon an integrin, $\alpha_3\beta_1$. The mechanism for integrin $\alpha_3\beta_1$ involvement in TLR2/1 signaling is through its role in endocytosis of the lipopeptide. Using inhibitors of endosomal acidification and maturation and physical tethering of the ligand, we show that the endocytosis of Pam_3CSK_4 is necessary for the complete TLR2/1-mediated pro-inflammatory cytokine response. We also show that TLR2/1 signaling from the endosome results in the induction of different inflammatory mediators than TLR2/1 signaling from the plasma membrane. We therefore have identified integrin $\alpha_3\beta_1$ as a novel regulator for the recognition of bacterial lipopeptides. We have also addressed an ongoing controversy regarding endosomal recognition of bacterial lipopeptides by demonstrating that TLR2/1 signals from within endosomal compartments as well as the plasma membrane, and that downstream responses may differ depending upon receptor localization. We propose that the regulation of endosomal TLR2/1 signaling by integrin $\alpha_3\beta_1$ serves as a mechanism for modulating inflammatory responses.

CHAPTER 2 RESULTS

Integrin $\alpha_3\beta_1$ mediates the U937 macrophage response to Pam_3CSK_4

To determine whether integrin $\alpha_3\beta_1$ cooperates with TLR2/1 signaling, we used short hairpin RNA (shRNA) to reduce the expression of integrin α_3 by 73% in U937 macrophages (Figure 2.1A). Specificity of the shRNA construct was confirmed by demonstrating that the shRNA did not significantly affect the expression of other integrin α chains or TLR2 (Figure 2.1B). U937 macrophages stably transduced with either non-targeting, control shRNA or integrin α_3-targeting shRNA were stimulated with the synthetic TLR2/1 ligand Pam_3CSK_4 under serum-free conditions. shRNA targeting integrin α_3 reduced the IL-6 response to Pam_3CSK_4 by 62% compared to the control shRNA construct ($p = 0.014$) (Figure 2.2A).

To confirm this finding, we tested the effects of antibody blocking of integrin $\alpha_3\beta_1$ on the response to Pam_3CSK_4. U937 macrophages were pre-treated with either control mouse ascites fluid (CMA) or an integrin $\alpha_3\beta_1$ function-inhibiting antibody (clone P1B5) in ascites fluid prior to stimulation with Pam_3CSK_4 under serum-free conditions. P1B5 specifically inhibits the function of integrin $\alpha_3\beta_1$ by preventing the interaction between integrin $\alpha_3\beta_1$ and its ligands (201, 214). Pre-treatment with P1B5 resulted in a 31% decrease in Pam_3CSK_4-induced IL-6 secretion compared to pre-treatment with CMA ($p = 0.014$) (Figure 2.2B). Taken together, these data suggest that integrin $\alpha_3\beta_1$ modulates TLR2/1 signaling in response to Pam_3CSK_4.

Figure 2.1

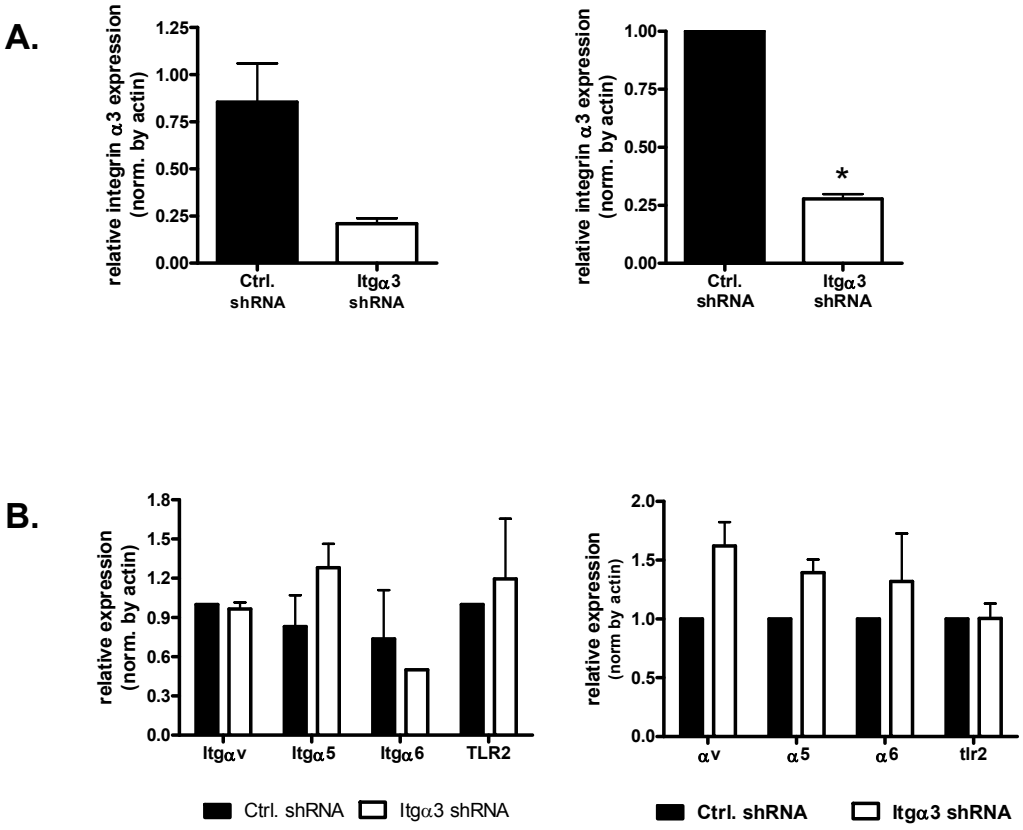

A.
relative integrin α3 expression (norm. by actin)
1.25
1.00
0.75
0.50
0.25
0.00
Ctrl. shRNA
Itgα3 shRNA
relative integrin α3 expression (norm. by actin)
1.00
0.75
0.50
0.25
0.00
*
Ctrl. shRNA
Itgα3 shRNA
B.
relative expression (norm. by actin)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
Itgαv
Itgα5
Itgα6
TLR2
Ctrl. shRNA
Itgα3 shRNA
relative expression (norm by actin)
2.0
1.5
1.0
0.5
0.0
αv
α5
α6
tlr2
Ctrl. shRNA
Itgα3 shRNA

Figure 2.1 shRNA targeting integrin α_3 specifically reduces the expression of integrin α_3

(A) U937 cells were stably transduced with integrin α_3-targeting shRNA (Itgα3 shRNA) or non-targeting shRNA (Ctrl. shRNA) and analyzed by qRT-PCR. On the left are the data from one representative experiment showing mean integrin α_3 expression compared to cells transduced with control shRNA (arbitrarily set to 1, error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments (error bars represent the S.E.M.). * p = 0.037. **(B)** U937 cells were stably transduced with integrin α_3-targeting shRNA (Itgα3 shRNA) or non-targeting shRNA (Ctrl. shRNA) and analyzed by qRT-PCR. On the left are the data from one representative experiment showing mean integrin or TLR2 expression compared to cells transduced with control shRNA (arbitrarily set to 1, error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments (error bars represent the S.E.M.). Normalized (norm.).

Figure 2.2

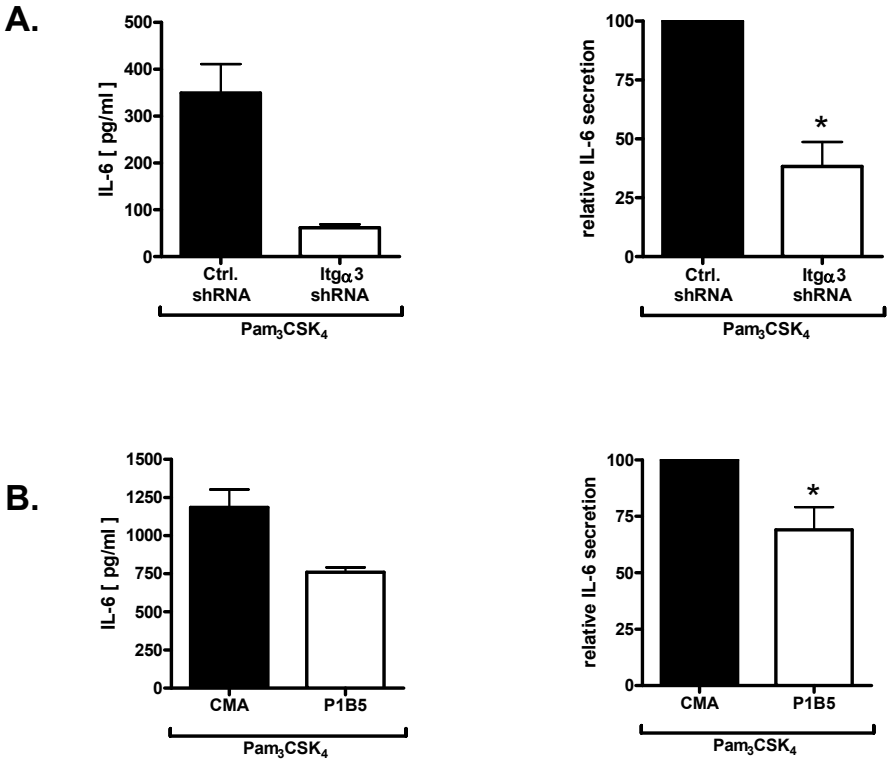

A.
IL-6 [pg/ml]
500
400
300
200
100
0
Ctrl. shRNA
Itgα3 shRNA
Pam3CSK4
relative IL-6 secretion
100
75
50
25
0
*
Ctrl. shRNA
Itgα3 shRNA
Pam3CSK4
B.
IL-6 [pg/ml]
1500
1250
1000
750
500
250
0
CMA
P1B5
Pam3CSK4
relative IL-6 secretion
100
75
50
25
0
*
CMA
P1B5
Pam3CSK4

Figure 2.2 Integrin $\alpha_3\beta_1$ mediates the U937 macrophage response to Pam_3CSK_4

(A) U937 cells stably transduced with integrin α_3-specific shRNA (Itgα3 shRNA) or non-targeting shRNA (Ctrl. shRNA) were stimulated with 100 ng/ml Pam_3CSK_4 for 6 hours under serum-free conditions. IL-6 secretion was measured by ELISA. On the left are the data from one representative experiment showing mean IL-6 secretion (error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to cells transduced with control shRNA (arbitrarily set to 100%, error bars represent the S.E.M.). Cells transduced with control shRNA secreted a mean of 350 pg/ml, and cells transduced with integrin α_3-targeting shRNA secreted a mean of 61 pg/ml. * $p = 0.014$. **(B)** U937 cells were treated with an integrin $\alpha_3\beta_1$ blocking antibody (P1B5) or control mouse ascites fluid (CMA) and stimulated with 100 ng/ml Pam_3CSK_4 for 6 hours under serum-free conditions. IL-6 secretion was measured by ELISA. On the left are the data from one representative experiment showing mean IL-6 secretion (error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to CMA-treated cells (arbitrarily set to 100%, error bars represent the S.E.M.). CMA-treated cells secreted a mean of 1,068 pg/ml, and P1B5-treated cells secreted a mean of 607 pg/ml. * $p = 0.014$.

Exogenous serum proteins do not enhance the role of integrin $\alpha_3\beta_1$ in the inflammatory response to Pam_3CSK_4

Previous work by another group showed that the addition of 1% fetal bovine serum (FBS) to cell culture media dramatically enhanced (by 8-fold) the pro-inflammatory cytokine response to bacterial lipopeptides. This was suggested to occur through integrin $\alpha_v\beta_3$-mediated recognition of vitronectin, its preferred ligand, which was bound to bacterial lipopeptides (65). To determine whether exogenous serum would enhance the role of integrin $\alpha_3\beta_1$ in facilitating TLR2/1 function, we stimulated U937 macrophages with Pam_3CSK_4 in the presence or absence of 1% FBS. Compared to U937 macrophages stimulated under serum-free condition, the addition of 1% FBS did not enhance the secretion of IL-6 (Figure 2.3A). Furthermore, the addition of exogenous serum did not affect the role of integrin $\alpha_3\beta_1$ in the response to Pam_3CSK_4. Indeed, macrophages transduced with integrin α_3 shRNA secreted similarly less IL-6 than control macrophages when stimulated either under serum-free conditions or in the presence of 1% serum ($p = 0.037$) (Figure 2.3B). These data suggest that, unlike integrin $\alpha_v\beta_3$, integrin $\alpha_3\beta_1$ does not require exogenous serum proteins to regulate the U937 macrophage response to Pam_3CSK_4.

Figure 2.3

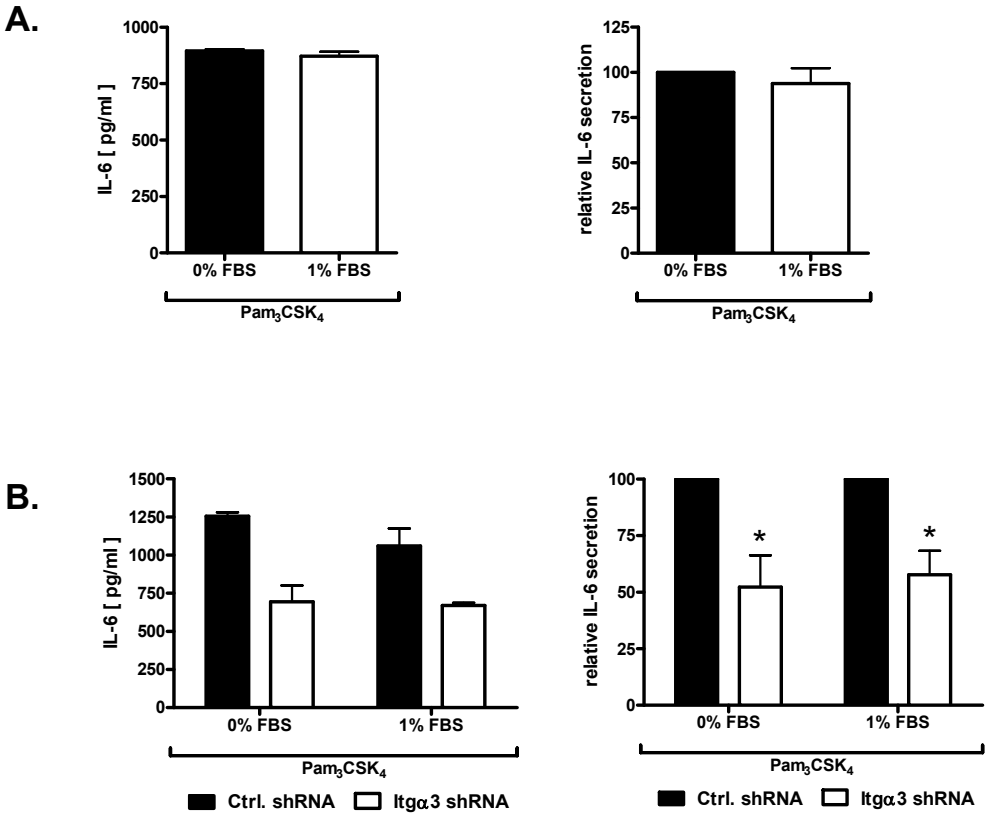

A.
IL-6 [pg/ml]
1000
750
500
250
0
0% FBS
1% FBS
Pam3CSK4
relative IL-6 secretion
125
100
75
50
25
0
0% FBS
1% FBS
Pam3CSK4
B.
IL-6 [pg/ml]
1500
1250
1000
750
500
250
0
0% FBS
1% FBS
Pam3CSK4
Ctrl. shRNA
Itgα3 shRNA
relative IL-6 secretion
100
75
50
25
0
*
*
0% FBS
1% FBS
Pam3CSK4
Ctrl. shRNA
Itgα3 shRNA

Figure 2.3 Exogenous serum proteins do not enhance the role of integrin $\alpha_3\beta_1$

(A) U937 cells were stimulated with 100 ng/ml Pam_3CSK_4 in the presence or absence of 1% FBS for 6 hours. IL-6 secretion was measured by ELISA. On the left are the data from one representative experiment showing mean IL-6 secretion (error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to cells stimulated under serum-free conditions (arbitrarily set to 100%, error bars represent the S.E.M.). Cells stimulated under serum-free conditions secreted a mean of 1,048 pg/ml, and cells stimulated in the presence of 1% FBS secreted a mean of 975 pg/ml. **(B)** U937 cells stably transduced with integrin α_3-specific shRNA (Itgα3 shRNA) or non-targeting shRNA (Ctrl. shRNA) were stimulated with 100 ng/ml Pam_3CSK_4 in the presence or absence of 1% FBS for 6 hours. IL-6 secretion was measured by ELISA. On the left are the data from one representative experiment showing mean IL-6 secretion (error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to cells transduced with control shRNA (arbitrarily set to 100%, error bars represent the S.E.M.). Under serum-free conditions, control cells secreted a mean of 1,048 pg/ml and cells transduced with integrin α_3-targeting shRNA secreted a mean of 545 pg/ml. When stimulated in the presence of 1% FBS, control cells secreted a mean of 974 pg/ml and cells transduced with integrin α_3-targeting shRNA secreted a mean of 556 pg/ml. * $p = 0.037$. Fetal bovine serum (FBS).

Integrin $\alpha_3\beta_1$ does not mediate association of Pam_3CSK_4 to U937 macrophages

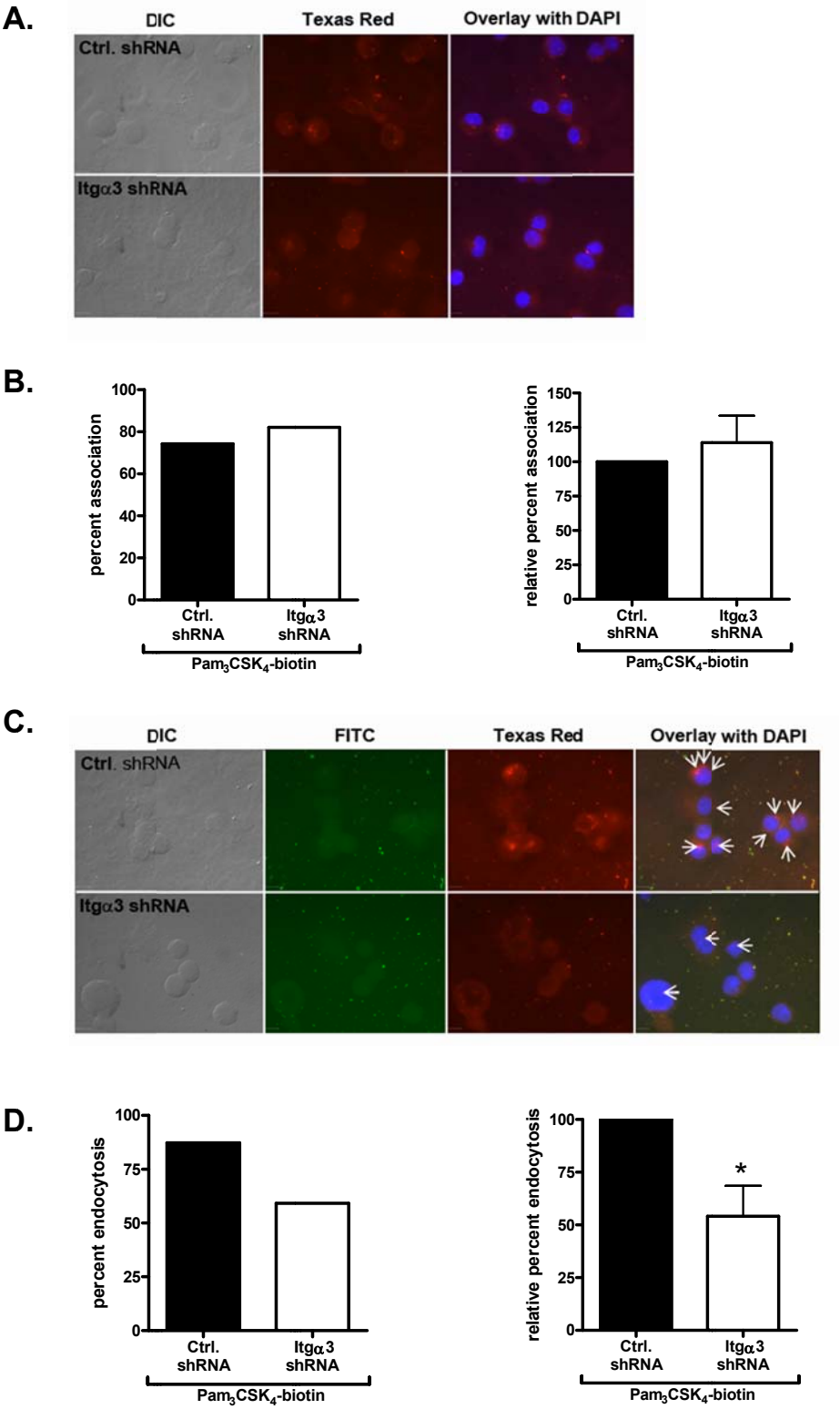
The interaction between integrin $\alpha_v\beta_3$ and vitronectin-lipopeptide complexes was proposed to mediate macrophage responses by facilitating clustering of TLR2/1 with the lipopeptides at the cell surface (65). To determine whether integrin $\alpha_3\beta_1$ affects TLR2/1 responses to Pam_3CSK_4 by mediating association of the lipopeptides with macrophages, Pam_3CSK_4-biotin was added to U937 macrophages transduced with control shRNA or integrin α_3-targeting shRNA. After 60 minutes, the macrophages were fixed, permeabilized, and examined by immunofluorescent microscopy using an anti-biotin antibody conjugated to Texas Red (Figure 2.4A). The percent association was determined by counting the subset of macrophages with Pam_3CSK_4-biotin associated, and expressing this number as a percentage of the total number of macrophages. No decrease in the association of Pam_3CSK_4-biotin to macrophages transduced with integrin α_3 targeted shRNA was observed (Figure 2.4A and B). These data suggest that, unlike integrin $\alpha_v\beta_3$, integrin $\alpha_3\beta_1$ is not involved in the association of Pam_3CSK_4 with macrophages.

Integrin $\alpha_3\beta_1$ mediates endocytosis of Pam_3CSK_4 in U937 macrophages

Integrins are known to be involved in the internalization of ligands such as ECM proteins and pathogens or their products (52, 182). To determine whether integrin $\alpha_3\beta_1$ participates in endocytosis of Pam_3CSK_4, we again employed immunofluorescent methods. In this experiment, U937 macrophages were

incubated with Pam_3CSK_4-biotin for 60 minutes, then fixed and stained with anti-biotin antibodies before (FITC-labeled) and after (Texas Red-labeled) permeabilization to distinguish Pam_3CSK_4-biotin on the surface of macrophages from that which had been internalized (Figure 2.4C). The percent endocytosis was determined by counting the subset of macrophages with which Pam_3CSK_4-biotin molecules was associated, and determining the fraction of these macrophages that had internalized the ligand. Knockdown of integrin α_3 resulted in a 46% decrease in the number of cells with internal Pam_3CSK_4-biotin ($p = 0.037$) (Figure 2.4C and D). These data demonstrate that integrin $\alpha_3\beta_1$ is necessary for the endocytosis of Pam_3CSK_4.

Figure 2.4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DIC
Texas Red
Overlay with DAPI
Ctrl. shRNA
Itgα3 shRNA
B.
percent association
100
80
60
40
20
0
Ctrl. shRNA
Itgα3 shRNA
Pam3CSK4-biotin
relative percent association
150
125
100
75
50
25
0
Ctrl. shRNA
Itgα3 shRNA
Pam3CSK4-biotin
C.
DIC
FITC
Texas Red
Overlay with DAPI
Ctrl. shRNA
Itgα3 shRNA
D.
percent endocytosis
100
75
50
25
0
Ctrl. shRNA
Itgα3 shRNA
Pam3CSK4-biotin
relative percent endocytosis
100
75
50
25
0
*
Ctrl. shRNA
Itgα3 shRNA
Pam3CSK4-biotin

Figure 2.4 Integrin $\alpha_3\beta_1$ mediates internalization, but not attachment, of Pam_3CSK_4

(A) U937 cells were stably transduced with integrin α_3-targeting shRNA (Itgα3 shRNA) or non-targeting shRNA (Ctrl. shRNA), stimulated with 5 μg/ml Pam_3CSK_4-biotin for 60 minutes, and fixed and stained for immunofluorescent microscopy. Pam_3CSK_4-biotin was detected by α-biotin antibodies conjugated to Texas Red. Scale bars, 10 μm. Data are representative of three independent experiments. **(B)** The association of Pam_3CSK_4-biotin with the cells was quantified by determining the percent association (the number of cells associated with Pam_3CSK_4-biotin divided by total cells). On the left are data from one representative experiment showing percent association. On the right are the combined data from three independent experiments showing relative percent association compared to cells transduced with control shRNA (arbitrarily set to 100%, error bars represent the S.E.M.). The mean percent association for control cells was 54.6%, and the mean percent association for cells transduced with integrin α_3-targeting shRNA was 60.6%. **(C)** U937 cells were stably transduced with integrin α_3-targeting shRNA (Itgα3 shRNA) or non-targeting shRNA (Ctrl. shRNA) and stimulated with 5 μg/ml Pam_3CSK_4-biotin for 60 minutes. The cells were fixed and stained for immunofluorescent microscopy using α-biotin antibodies before (FITC) or after (Texas Red) permeabilization of the cells. White arrows indicate internalized Pam_3CSK_4-biotin. Scale bars, 10μm. Data are representative of three independent experiments. **(D)** The endocytosis of Pam_3CSK_4-biotin was quantified by determining the percent endocytosis (the

number of cells with internalized Pam_3CSK_4-biotin divided by number of cells with Pam_3CSK_4-biotin associated). On the left are data from one representative experiment showing percent endocytosis. On the right are the combined data from three independent experiments showing relative percent endocytosis compared to cells transduced with control shRNA (arbitrarily set to 100%, error bars represent the S.E.M.). The mean percent endocytosis for control cells was 79.3%, and the mean percent endocytosis for cells transduced with integrin α_3-targeting shRNA was 42.9%. * $p = 0.037$. Differential interface contrast (DIC), 4',6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC).

Pam_3CSK_4 induces signaling through TLR2/1 from endosomal compartments, and is internalized through clathrin-mediated endocytosis

Having shown that integrin $\alpha_3\beta_1$ mediates endocytosis of Pam_3CSK_4 into sub-cellular compartments, we next sought to determine whether this internalization is important for the inflammatory response to the ligand. To determine whether TLR2 and Pam_3CSK_4 are localized together within the macrophages, we examined co-localization by confocal microscopy. Pam_3CSK_4-rhodamine was incubated with U937 macrophages for 20 min and subsequently fixed and stained with anti-TLR2 antibodies, followed by a secondary anti-mouse antibody conjugated to Alexa Fluor 488 (Figure 2.5). Macrophages were visualized by confocal microscopy. For simultaneous green and red channel imaging, the multitracking function was utilized, and each laser was activated one at a time, ensuring that no cross-talk occurred between the two fluorochromes. Z plane images of 0.7 μm were captured using the 63X oil objective lens, and analyzed using the Leica Confocal Software. As shown in Figure 2.5, Pam_3CSK_4 and TLR2 co-localize in intracellular compartments.

Figure 2.5

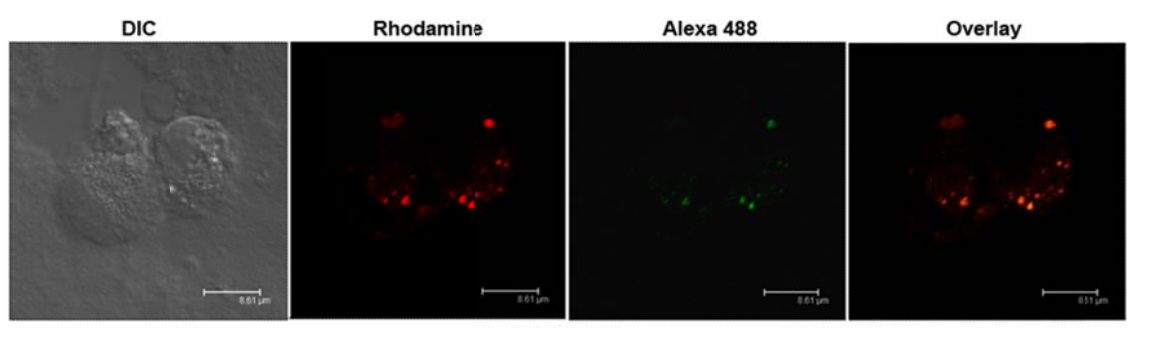


Figure 2.5 Pam_3CSK_4 co-localizes with TLR2 intracellularly

U937 cells were stimulated with 5 μg/ml Pam_3CSK_4-rhodamine for 20 minutes. The cells were fixed and stained for immunofluorescent microscopy using anti-TLR2 antibodies and secondary antibodies conjugated to Alexa Fluor 488. Data are representative of three independent experiments. Images show one representative Z plane of 0.7 μm thickness. Scale bars, 8.61 μm. Differential interface contrast (DIC)

To determine whether intracellular TLR2/1 is capable of signaling in response to Pam_3CSK_4, we pre-treated macrophages with inhibitors of endosomal acidification and maturation. We first tested the effects of the vacuolar-ATP-ase (v-ATP-ase) inhibitors concanamycin A and bafilomycin A1. Pre-treatment of macrophages with these inhibitors resulted in significant 53% and 37% decreases in IL-6 secretion ($p = 0.037$) (Figure 2.6A). To further confirm the importance of endosomal acidification, and to rule out a non-specific effect of v-ATPase inhibitors, we also determined the effects of monensin, an antibiotic ionophore, which acts as a Na+/K+ antiporter and inhibits endosomal acidification through a different mechanism. Pre-treatment with monensin also reduced IL-6 secretion by 38% ($p = 0.037$) (Figure 2.6B).

A caveat to the use of monensin is that it is a known inhibitor of intracellular protein transport. Although we used monensin at concentrations that have not been reported to inhibit protein transport to a significant degree (203), we confirmed our IL-6 ELISA measurements by examining mRNA transcript levels. Quantitative reverse transcriptase PCR (qRT-PCR) analysis of IL-6 transcript confirmed that pre-treatment with monensin reduced expression of this cytokine 32% in Pam_3CSK_4 stimulated macrophages (Figure 2.7). Taken together, these data suggest that endocytosis and subsequent endosomal acidification are important for the Pam_3CSK_4-induced IL-6 response.

Figure 2.6

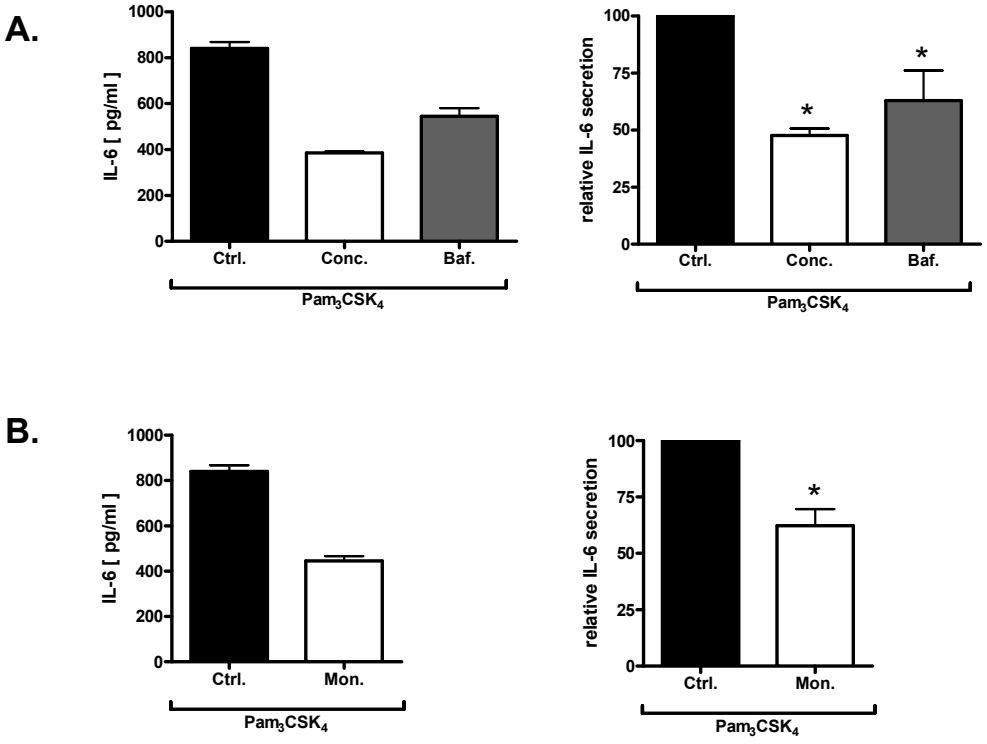

A.
1000
800
600
400
200
0
IL-6 [pg/ml]
Ctrl.
Conc.
Baf.
Pam3CSK4
100
75
50
25
0
relative IL-6 secretion
*
*
Ctrl.
Conc.
Baf.
Pam3CSK4
B.
1000
800
600
400
200
0
IL-6 [pg/ml]
Ctrl.
Mon.
Pam3CSK4
100
75
50
25
0
relative IL-6 secretion
*
Ctrl.
Mon.
Pam3CSK4

Figure 2.6 Pam_3CSK_4 induces signaling through TLR2/1 from endosomal compartments

(A) U937 cells were treated with 100 ng/ml concanamycin A (Conc.), 500 μM bafilomycin A1 (Baf.), or control (Ctrl.), and stimulated with 100 ng/ml Pam_3CSK_4 for 6 hours under serum-free conditions. IL-6 secretion was measured by ELISA. On the left are the data from one representative experiment showing mean IL-6 secretion (error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to control cells (arbitrarily set to 100%, error bars represent the S.E.M.). Control cells secreted a mean of 670 pg/ml, concanamycin A-treated cells secreted a mean of 320 pg/ml, and bafilomycin A1-treated cells secreted a mean of 430 pg/ml. * $p = 0.037$. **(B)** U937 cells were treated with 1 μM monensin (Mon.) or control (Ctrl.), and stimulated with 100 ng/ml Pam_3CSK_4 for 6 hours under serum-free conditions. IL-6 secretion was measured by ELISA. On the left are the data from one representative experiment showing mean IL-6 secretion (error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to control cells (arbitrarily set to 100%, error bars represent the S.E.M.). Control cells secreted a mean of 670 pg/ml, and monensin-treated cells secreted a mean of 420 pg/ml. * $p = 0.037$.

Figure 2.7

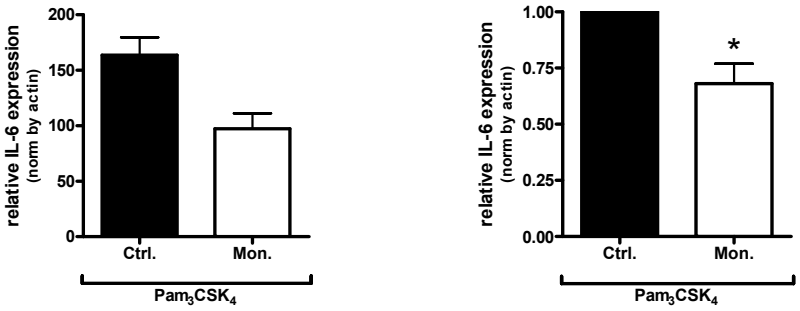


Figure 2.7 Monensin reduces expression of IL-6 mRNA in response to Pam_3CSK_4

U937 cells were treated with 1 μM monensin (Mon.) or control (Ctrl.), and stimulated with 100 ng/ml Pam_3CSK_4 for 6 hours under serum-free conditions. IL-6 expression was analyzed by qRT-PCR. On the left are the data from one representative experiment showing mean IL-6 expression compared to cells transduced with control shRNA (arbitrarily set to 1, error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments (error bars represent the S.E.M.). * $p = 0.037$. Normalized (norm.).

A previous study suggested that Pam_3CSK_4-ovalbumin (OVA) conjugates are endocytosed by dendritic cells through a clathrin-dependent mechanism. This study did not address whether clathrin-mediated uptake of Pam_3CSK_4-OVA was through interaction with the lipopeptide or the OVA component (115). To determine whether endocytosis of Pam_3CSK_4 is dependent on clathrin, we tested the addition of chlorpromazine (CPZ), an inhibitor of clathrin-mediated endocytosis (140). CPZ had a significant effect on the response to Pam_3CSK_4, reducing the secretion of IL-6 in U937 macrophages by 49% ($p = 0.037$) (Figure 2.8). These data suggest that endocytosis of Pam_3CSK_4 is clathrin-mediated.

Figure 2.8

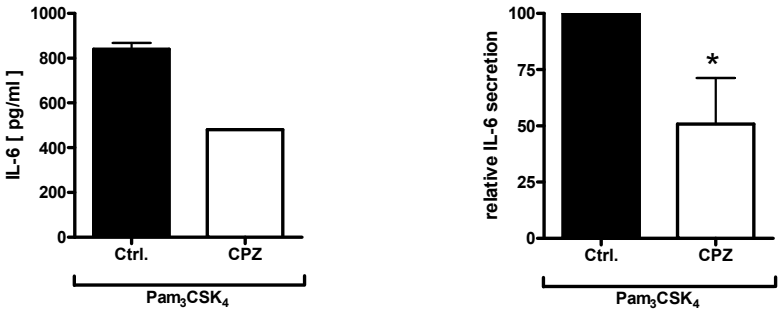


Figure 2.8 Pam_3CSK_4 is internalized through clathrin-mediated endocytosis

U937 cells were treated with 5 µM chlorpromazine (CPZ) or control (Ctrl.) and stimulated with 100 ng/ml Pam_3CSK_4 for 6 hours under serum-free conditions. IL-6 secretion was measured by ELISA. On the left are the data from one representative experiment showing mean IL-6 secretion (error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to control cells (arbitrarily set to 100%, error bars represent the S.E.M.). Control cells secreted a mean of 670 pg/ml and CPZ-treated cells secreted a mean of 340 pg/ml. * p = 0.037.

Because all chemical inhibitors may have off-target effects, we further confirmed the importance of endocytosis on Pam_3CSK_4-induced IL-6 by immobilizing Pam_3CSK_4-biotin to streptavidin plates to prevent internalization. Pam_3CSK_4-biotin was bound to streptavidin plates overnight and washed prior to the addition of U937 macrophages. As compared to macrophages stimulated with free Pam_3CSK_4-biotin, macrophages stimulated with plate-bound Pam_3CSK_4-biotin secreted 56% less IL-6 ($p = 0.037$) (Figure 2.9A). In addition, to ascertain whether the amounts of Pam_3CSK_4-biotin were similar in the "plate-bound" versus "soluble" stimulation methods, we used a second stimulation method. We first blocked the streptavidin wells with biotin-HRP or control. We then added U937 macrophages and Pam_3CSK_4-biotin to blocked and non-blocked wells simultaneously. In this experiment, a portion of the Pam_3CSK_4 in the non-blocked wells would be expected to bind to streptavidin on the plate, thus reducing the amount of free lipopeptide for endocytosis. We observed a 48% decrease in IL-6 production in the non-blocked wells compared to the blocked wells ($p = 0.037$) (Figure 2.9B). In each experimental condition, macrophages incubated with immobilized Pam_3CSK_4-biotin secreted higher levels of TNF-α, suggesting that the immobilization of the ligand does not inhibit the inherent ability of the cells to respond to the ligand (Figure 2.9C). Thus, these data confirm the role of endocytosis of Pam_3CSK_4 in inducing TLR2/1-dependent pathways from sub-cellular compartments.

Figure 2.9

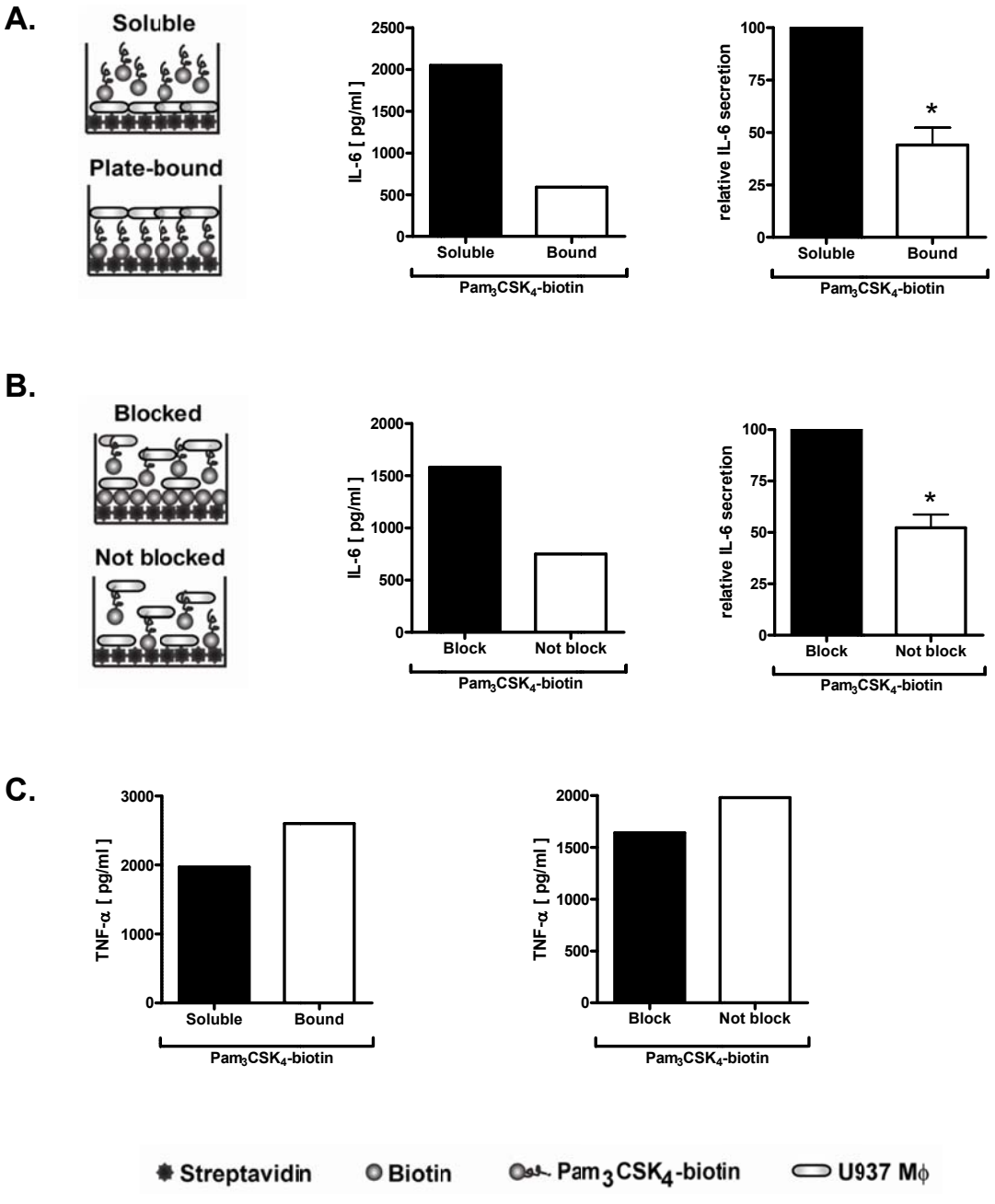

A.
Soluble
Plate-bound
IL-6 [pg/ml]
2500
2000
1500
1000
500
0
Soluble
Bound
Pam3CSK4-biotin
relative IL-6 secretion
100
75
50
25
0
*
Soluble
Bound
Pam3CSK4-biotin
B.
Blocked
Not blocked
IL-6 [pg/ml]
2000
1500
1000
500
0
Block
Not block
Pam3CSK4-biotin
relative IL-6 secretion
100
75
50
25
0
*
Block
Not block
Pam3CSK4-biotin
C.
TNF-α [pg/ml]
3000
2000
1000
0
Soluble
Bound
Pam3CSK4-biotin
TNF-α [pg/ml]
2000
1500
1000
500
0
Block
Not block
Pam3CSK4-biotin
Streptavidin
Biotin
Pam3CSK4-biotin
U937 Mϕ

Figure 2.9 The IL-6 response is dependent upon internalization of Pam_3CSK_4

(A) U937 cells were stimulated with 5 μg/ml of either soluble Pam_3CSK_4-biotin (Soluble) or Pam_3CSK_4-biotin immobilized on streptavidin plates (Bound) for 6 hours under serum-free conditions. IL-6 secretion was measured by ELISA. On the left is a schematic of the experimental design. In the center are the data from one representative experiment showing IL-6 secretion. On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to cells stimulated with soluble Pam_3CSK_4-biotin (arbitrarily set to 100%, error bars represent the S.E.M.). Cells stimulated with soluble Pam_3CSK_4-biotin secreted a mean of 1,456 pg/ml, and cells stimulated with plate-bound Pam_3CSK_4-biotin secreted a mean of 620 pg/ml. * p = 0.037. **(B)** U937 cells were stimulated with 5 μg/ml of soluble Pam_3CSK_4-biotin in either non-blocked streptavidin plates (Not block) or streptavidin plates blocked with biotin-HRP (Block) for 6 hours under serum-free conditions. On the left is a schematic of the experimental design. In the center are the data from one representative experiment showing IL-6 secretion. On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to cells stimulated in blocked wells (arbitrarily set to 100%, error bars represent the S.E.M.). Cells stimulated in blocked wells secreted a mean of 1,690 pg/ml and cells stimulated in non-blocked wells secreted a mean of 895 pg/ml. * p = 0.037. **(C)** Data shown are TNF-α secretion under each experimental design from one experiment. Macrophages (Mϕ).

TLR2/1 transduces signals from the endosome for the induction of IFN-α1

Endosomal TLR2/1 has been shown to induce IFN-I, specifically IFN-β, in response to viral and bacterial ligands (11, 51). While two independent studies have shown that endosomal TLR2 induces IFN-β (11, 111), we did not observe the induction of IFN-β in U937 macrophages at either 6 hrs or 16 hrs post stimulation (Figure 2.10A). However, we sought to determine whether endosomal TLR2 could induce other IFN-I. We examined the role of Pam_3CSK_4 stimulation on the induction of IFN-α1, the major IFN-α subtype elicited by human plasmacytoid dendritic cells (pDCs) (143). U937 macrophages were stimulated with Pam_3CSK_4 for 6 and 16 hours in the presence or absence of the endosomal acidification inhibitors concanamycin A and monensin. Inhibition of endosomal acidification had a dramatic effect on the expression of IFN-α1, reducing the transcript levels by 84% for concanamycin-treated macrophages and 88% for monensin-treated macrophages ($p = 0.037$) (Figure 2.10B). These data demonstrate that Pam_3CSK_4 induces an interferon response in U937 macrophages, and that this interferon response requires TLR2/1 signaling from endosomal compartments.

Figure 2.10

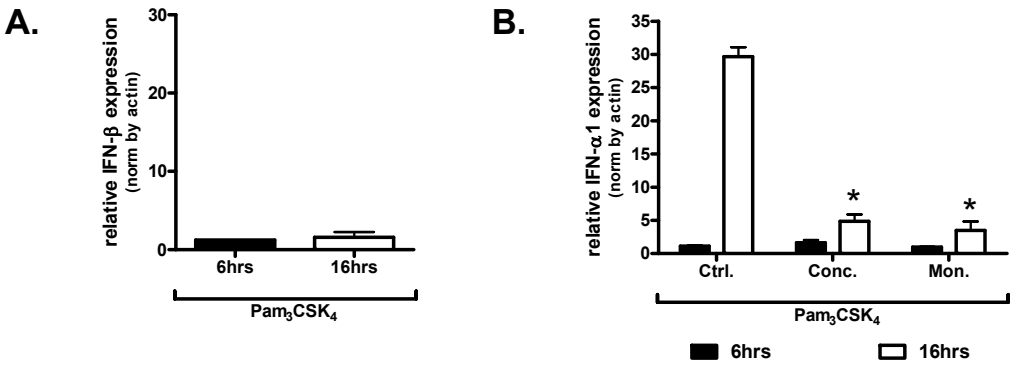


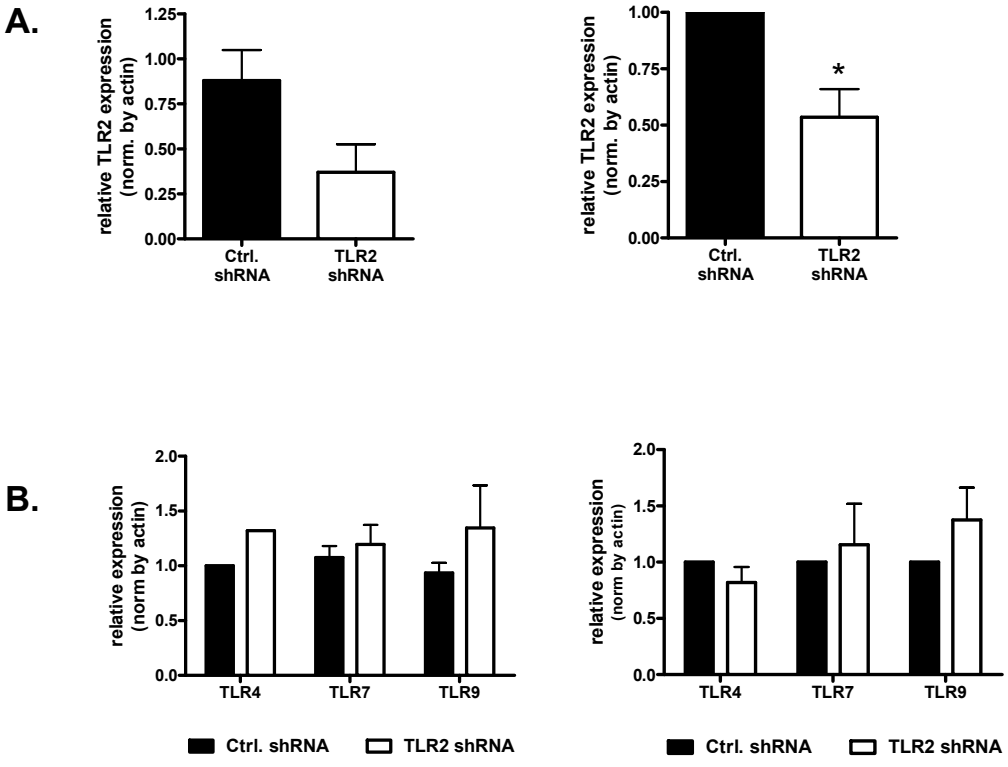
Figure 2.10 TLR2/1 transduces signals from the endosome for the induction of IFN-α1

(A) U937 cells were treated with 100ng/ml Pam_3CSK_4 under serum-free conditions for the indicated times. Expression of IFN-β was measured by qRT-PCR. Shown are the combined data from three independent experiments showing mean IFN-β expression relative to unstimulated cells (arbitrarily set to 1 (not shown), error bars represent S.E.M.). **(B)** U937 cells were treated with 100 ng/ml concanamycin A (Conc.), 1 μM monensin (Mon.), or control (Ctrl.), and stimulated with 100 ng/ml Pam_3CSK_4 under serum-free conditions for the indicated times. Expression of IFN-α1 was measured by qRT-PCR. Shown are the combined data from three independent experiments showing mean IFN-α1 expression relative to unstimulated cells (arbitrarily set to 1 (not shown), error bars represent S.E.M.). * $p = 0.037$. Normalized (norm.), Hours (hrs).

TLR2 mediates the inflammatory cytokine response to *B. burgdorferi* in U937 macrophages

We have so far demonstrated that integrin $\alpha_3\beta_1$ mediates the secretion of IL-6 in response to the synthetic TLR2/1 ligand Pam_3CSK_4 by regulating endocytosis of the ligand and facilitating its recognition by TLR2/1 from within endosomal compartments. To confirm the role of integrin $\alpha_3\beta_1$ and sub-cellular signaling by TLR2/1 in the recognition of lipoproteins presented in the context of a bacterial membrane, we stimulated U937 macrophages with a bacterium that expresses numerous lipoproteins, *B. burgdorferi*. It has previously been reported that TLR2/1 plays the major role in the induction of the inflammatory response to *B. burgdorferi* in macrophages (86, 188, 215, 216). We first determined the degree to which TLR2 is responsible for the IL-6 response to *B. burgdorferi* in U937 macrophages. Expression of TLR2 mRNA was reduced by 47% in U937 macrophages by use of shRNA (Figure 2.11A). Specificity of the shRNA was confirmed by demonstrating that the construct did not affect the expression of other TLRs (Figure 2.11B). Decreased expression of TLR2 reduced the secretion of IL-6 in response to *B. burgdorferi* by 70% ($p = 0.037$) (Figure 2.12)**.** These data suggest that signaling through TLR2 is responsible for the majority of *B. burgdorferi*-induced IL-6 secretion in U937 macrophages.

Figure 2.11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0.75
0.50
0.25
0.00
Ctrl. shRNA
TLR2 shRNA
relative TLR2 expression (norm. by actin)
1.00
0.75
0.50
0.25
0.00
*
Ctrl. shRNA
TLR2 shRNA
B.
relative expression (norm by actin)
2.0
1.5
1.0
0.5
0.0
TLR4
TLR7
TLR9
Ctrl. shRNA
TLR2 shRNA
relative expression (norm by actin)
2.0
1.5
1.0
0.5
0.0
TLR4
TLR7
TLR9
Ctrl. shRNA
TLR2 shRNA

Figure 2.11 shRNA targeting TLR2 specifically reduces the expression of TLR2

(A) U937 cells were stably transduced with TLR2-targeting shRNA (TLR2 shRNA) or non-targeting shRNA (Ctrl. shRNA) and analyzed by qRT-PCR. On the left are the data from one representative experiment showing mean TLR2 expression compared to cells transduced with control shRNA (arbitrarily set to 1, error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments (error bars represent the S.E.M.). * $p = 0.037$. **(B)** U937 cells were stably transduced with TLR2-targeting shRNA (TLR2 shRNA) or non-targeting shRNA (Ctrl. shRNA) and analyzed by qRT-PCR. On the left are the data from one representative experiment showing mean TLR expression compared to cells transduced with control shRNA (arbitrarily set to 1, error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments (error bars represent the S.E.M.). Normalized (norm.).

Figure 2.12

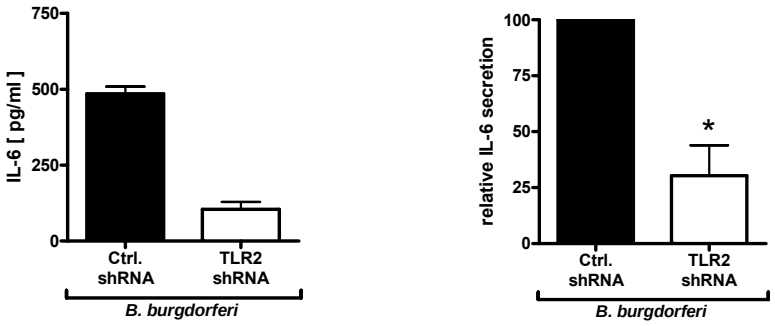


Figure 2.12 TLR2 mediates the inflammatory cytokine response to *B. burgdorferi* in U937 macrophages

U937 cells were stably transduced with TLR2-specific shRNA (TLR2 shRNA) or non-targeting shRNA (Ctrl. shRNA) and stimulated with *B. burgdorferi* MOI 10 for 6 hours under serum-free conditions. IL-6 secretion was measured by ELISA. On the left are the data from one representative experiment showing mean IL-6 secretion (error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to cells transduced with control shRNA (arbitrarily set to 100%, error bars represent the S.E.M.). Cells transduced with control shRNA secreted a mean of 553 pg/ml, and cells transduced with TLR2-targeting shRNA secreted a mean of 142 pg/ml. * $p = 0.037$.

Integrin $\alpha_3\beta_1$ mediates the inflammatory response to *B. burgdorferi* in U937 macrophages

We have previously shown that integrin $\alpha_3\beta_1$ plays an important role in mediating the inflammatory response to *B. burgdorferi* in human chondrocyte cells (18). However, most of the work by our lab and others examining the inflammatory response to *B. burgdorferi* is performed in macrophages, not in chondrocyte cells. Thus, whether the role of integrin $\alpha_3\beta_1$ in chondrocyte cells is relevant to the inflammatory responses observed in macrophages remains unknown. To determine whether integrin $\alpha_3\beta_1$ regulates the inflammatory response in our macrophage model of infection, we tested the effects of integrin α_3-targeting shRNA and antibody blocking of integrin $\alpha_3\beta_1$ on the U937 macrophage response to *B. burgdorferi.* shRNA targeting integrin α_3 reduced the IL-6 response to *B. burgdorferi* by 47% ($p = 0.014$) (Figure 2.13A). Pre-treatment with the integrin $\alpha_3\beta_1$ blocking antibody P1B5 resulted in a 68% decrease ($p = 0.014$) in *B. burgdorferi*-induced IL-6 secretion compared to pre-treatment with CMA (Figure 2.13B), confirming the findings in the shRNA experiments. These data demonstrate that integrin $\alpha_3\beta_1$ participates in the inflammatory cytokine response to *B. burgdorferi* not only in chondrocytes, but also in macrophages, and confirms that the role of integrin $\alpha_3\beta_1$ previously described by our lab is also important in the more common macrophage cell model of *B. burgdorferi* infection.

Figure 2.13

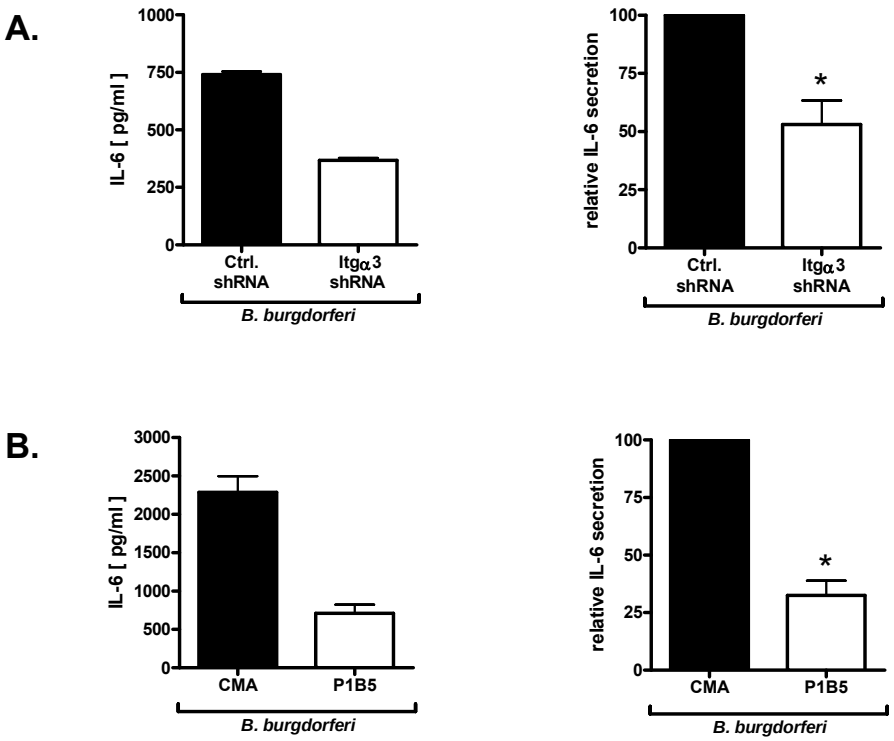

A.
1000
750
500
250
0
IL-6 [pg/ml]
Ctrl. shRNA
Itgα3 shRNA
B. burgdorferi
100
75
50
25
0
relative IL-6 secretion
*
Ctrl. shRNA
Itgα3 shRNA
B. burgdorferi
B.
3000
2500
2000
1500
1000
500
0
IL-6 [pg/ml]
CMA
P1B5
B. burgdorferi
100
75
50
25
0
relative IL-6 secretion
*
CMA
P1B5
B. burgdorferi

Figure 2.13 Integrin $\alpha_3\beta_1$ mediates the inflammatory response to *B. burgdorferi* in U937 macrophages

(A) U937 cells were stably transduced with integrin α_3-specific shRNA (Itgα3 shRNA) or non-targeting shRNA (Ctrl. shRNA), and stimulated with *B. burgdorferi* MOI 10 for 6 hours under serum-free conditions. IL-6 secretion was measured by ELISA. On the left are the data from one representative experiment showing mean IL-6 secretion (error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to cells transduced with control shRNA (arbitrarily set to 100%, error bars represent the S.E.M.). Cells transduced with control shRNA secreted a mean of 560 pg/ml, and cells transduced with integrin α_3-targeting shRNA secreted a mean of 310 pg/ml. * p = 0.014. **(B)** U937 cells were treated with an integrin $\alpha_3\beta_1$ blocking antibody (P1B5) or control mouse ascites fluid (CMA) and stimulated with *B. burgdorferi* MOI 10 for 6 hours under serum-free conditions. IL-6 secretion was measured by ELISA. On the left are the data from one representative experiment showing mean IL-6 secretion (error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to CMA-treated cells (arbitrarily set to 100%, error bars represent the S.E.M.). CMA-treated cells secreted a mean of 1,500 pg/ml, and P1B5-treated cells secreted a mean of 560 pg/ml. * p = 0.014.

Integrin $\alpha_3\beta_1$ does not mediate attachment, but does mediate endocytosis of *B. burgdorferi* by U937 macrophages

To determine whether integrin $\alpha_3\beta_1$ regulates the inflammatory response to *B. burgdorferi* by regulating association with the macrophages and subsequent endocytosis, U937 macrophages were stably transduced with shRNA targeting integrin α_3 or control shRNA prior to stimulation with the spirochetes. At 60 minutes, the macrophages were fixed and visualized by immunofluorescent microscopy using an anti-*B. burgdorferi* polyclonal antibody and fluorescently labeled secondary antibodies. Integrin α_3-targeting shRNA did not reduce the percent association (Figure 2.14A and B). However, integrin α_3 shRNA did inhibit endocytosis of the organism, decreasing the percent endocytosis by 53% ($p = 0.037$) (Figure 2.14A and C). These results suggest that, like its role in the response to Pam_3CSK_4, integrin $\alpha_3\beta_1$ regulates the endocytosis, but not the association, of *B. burgdorferi* in U937 macrophages.

Figure 2.14

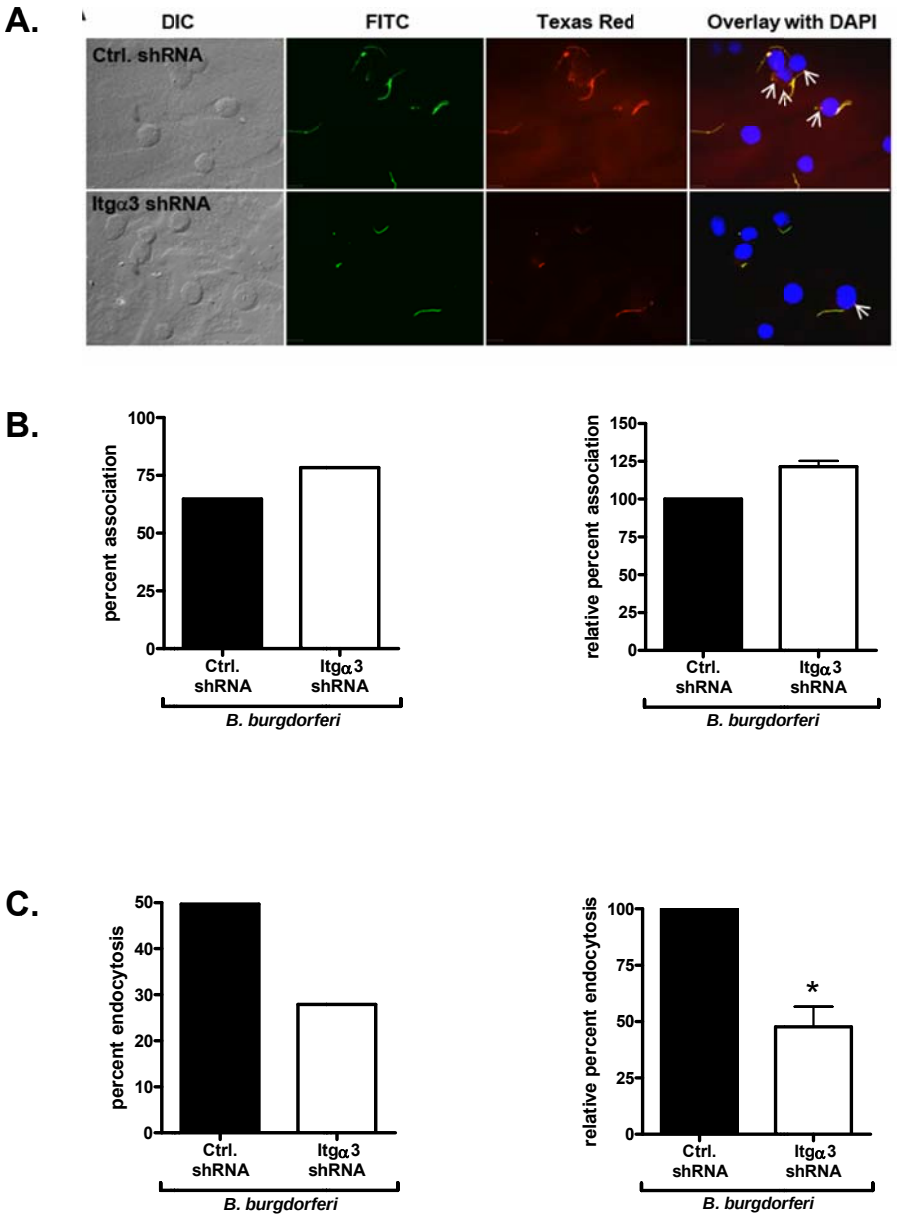

A.
DIC
FITC
Texas Red
Overlay with DAPI
Ctrl. shRNA
Itgα3 shRNA
B.
percent association
100
75
50
25
0
Ctrl. shRNA
Itgα3 shRNA
B. burgdorferi
relative percent association
150
125
100
75
50
25
0
Ctrl. shRNA
Itgα3 shRNA
B. burgdorferi
C.
percent endocytosis
50
40
30
20
10
0
Ctrl. shRNA
Itgα3 shRNA
B. burgdorferi
relative percent endocytosis
100
75
50
25
0
*
Ctrl. shRNA
Itgα3 shRNA
B. burgdorferi

Figure 2.14 Integrin $\alpha_3\beta_1$ mediates endocytosis of *B. burgdorferi*

(A) U937 macrophages were stably transduced with integrin α_3-specific shRNA (Itgα3 shRNA) or non-targeting shRNA (Ctrl. shRNA), stimulated with *B. burgdorferi* MOI 10 for 60 minutes under serum-free conditions. The cells were fixed and stained for immunofluorescent microscopy using anti-*B. burgdorferi* antibodies and fluorescently tagged secondary antibodies before (FITC) or after (Texas Red) permeabilization of the cells. White arrows indicate internalized *B. burgdorferi*. Scale bars, 10μm. Data are representative of three independent experiments. **(B)** The association of *B. burgdorferi* with the macrophages was quantified by determining the percent association (the number of cells associated with *B. burgdorferi* divided by the total number of cells). On the left are the data from one representative experiment showing percent association. On the right are the combined data from three independent experiments showing relative percent association compared to cells transduced with control shRNA (arbitrarily set to 100%, error bars represent the S.E.M.). The mean percent association for control cells was 51.1%, and the mean percent association for cells transduced with integrin α_3-targeting shRNA was 62.2%. **(C)** The endocytosis of *B. burgdorferi* was quantified by determining the percent endocytosis (the number of cells with *B. burgdorferi* internalized divided by the number of cells with *B. burgdorferi* associated). On the left are the data from one representative experiment showing percent endocytosis. On the right are the combined data from three independent experiments showing relative percent endocytosis compared to cells transduced with control shRNA (arbitrarily set to 100%, error

bars represent the S.E.M.). The mean percent endocytosis for control cells was 41.8%, and the mean percent endocytosis for cells transduced with integrin α_3-targeting shRNA was 20.1%. * $p = 0.037$. Differential interface contrast (DIC), 4',6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC).

Induction of inflammatory cytokines by *B. burgdorferi* occurs downstream of endocytosis and endosomal maturation

To determine whether acidification and endosomal maturation is important in the inflammatory response to *B. burgdorferi*, we tested the inhibitors used for the above studies with Pam_3CSK_4. The addition of either concanamycin A, bafilomycin A1 or monensin to U937 macrophages prior to the stimulation with *B. burgdorferi* inhibited the induction of IL-6 by 56%, 30% and 40%, respectively ($p = 0.037$) (Figure 2.15A and B). Monensin ELISA data was again confirmed by qRT-PCR. The IL-6 transcript was reduced 51% upon monensin pre-treatment of *B. burgdorferi*-stimulated macrophages (Figure 2.16). These studies support the concept that endosomal maturation and bacterial digestion within the endosome are important in eliciting a complete pro-inflammatory response to *B. burgdorferi*.

Figure 2.15

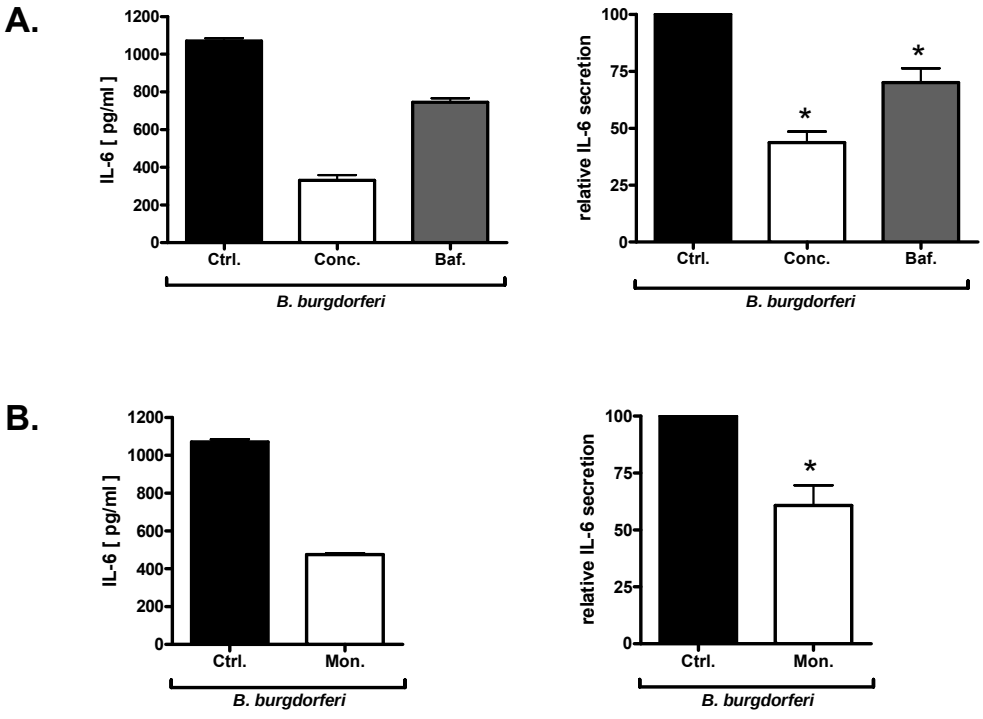

A.
IL-6 [pg/ml]
1200
1000
800
600
400
200
0
Ctrl.
Conc.
Baf.
B. burgdorferi
relative IL-6 secretion
100
75
50
25
0
*
*
Ctrl.
Conc.
Baf.
B. burgdorferi
B.
IL-6 [pg/ml]
1200
1000
800
600
400
200
0
Ctrl.
Mon.
B. burgdorferi
relative IL-6 secretion
100
75
50
25
0
*
Ctrl.
Mon.
B. burgdorferi

Figure 2.15 Induction of IL-6 by *B. burgdorferi* occurs downstream of endocytosis and endosomal maturation

(A) U937 cells were treated with 100 ng/ml concanamycin A (Conc.), 500 μM bafilomycin A1 (Baf.), or control (Ctrl.) and stimulated with *B. burgdorferi* MOI 10 for 6 hours under serum-free conditions. IL-6 secretion was measured by ELISA. On the left are the data from one representative experiment showing mean IL-6 secretion (error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to control cells (arbitrarily set to 100%, error bars represent the S.E.M.). Control cells secreted a mean of 955 pg/ml, concanamycin A-treated cells secreted a mean of 437 pg/ml, and bafilomycin A1-treated cells secreted a mean of 680 pg/ml. * $p = 0.037$. **(B)** U937 cells were treated with 1 μM monensin (Mon.) or control (Ctrl.), and stimulated with *B. burgdorferi* MOI 10 for 6 hours under serum-free conditions. IL-6 secretion was measured by ELISA. On the left are the data from one representative experiment showing mean IL-6 secretion (error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to control cells (arbitrarily set to 100%, error bars represent the S.E.M.). Control cells secreted a mean of 955 pg/ml and monensin-treated cells secreted a mean of 567 pg/ml. * $p = 0.037$.

Figure 2.16

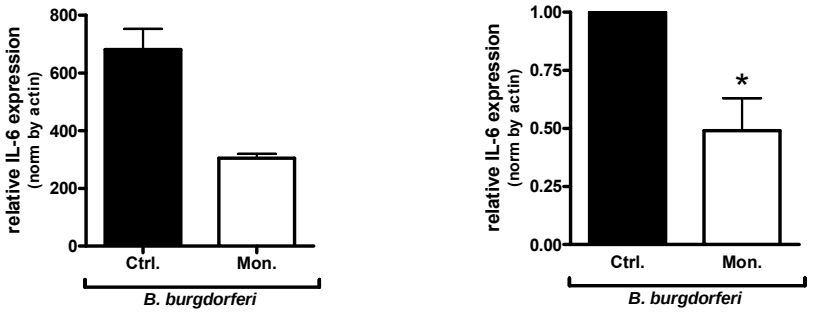


Figure 2.16 Monensin reduces expression of IL-6 mRNA in response to *B. burgdorferi*

U937 cells were treated with 1 μM monensin (Mon.) or control (Ctrl.), and stimulated with *B. burgdorferi* at MOI 10 for 6 hours under serum-free conditions. IL-6 expression was analyzed by qRT-PCR. On the left are the data from one representative experiment showing mean IL-6 expression compared to control cells (arbitrarily set to 1, error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments (error bars represent the S.E.M.). * p = 0.037. Normalized (norm.).

CHAPTER 3

THE ROLES OF TLR2 AND CD14 IN THE ENDOCYTOSIS OF AND INFLAMMATORY RESPONSE TO PAM_3CSK_4

CHAPTER 3 ABSTRACT

We have identified integrin $\alpha_3\beta_1$ as a co-receptor for TLR2/1. The mechanism of cooperation between integrin $\alpha_3\beta_1$ and TLR2/1 involves integrin $\alpha_3\beta_1$-mediated endocytosis of bacterial lipopeptides, which facilitates the recognition of these lipopeptides and initiation of TLR2/1 signaling from endosomal compartments. In addition to integrin $\alpha_3\beta_1$, TLR2/1 cooperates with other known co-receptors, such as CD14. However, the mechanism of cooperation between CD14 and TLR2/1 are not well understood. In ongoing work, our laboratory is interested in understanding whether TLR2/1 functions in any capacity in addition to signaling, and also in elucidating the mechanisms by which CD14 cooperates with TLR2/1. Using shRNA and fluorescent microscopy, we show that TLR2 does not participate in the endocytosis of its ligands. We also present data which suggests that, in addition to integrin $\alpha_3\beta_1$, CD14 may participate in the endocytosis of bacterial lipopeptides. These preliminary data, taken together, suggest that TLR2/1 may function primarily as a signaling molecule, with integrin $\alpha_3\beta_1$ and CD14 cooperating to mediate endocytosis of bacterial lipopeptides.

CHAPTER 3 RESULTS

TLR2 mediates the U937 macrophage response to Pam_3CSK_4

The TLR2/1 heterodimer is the known receptor for Pam_3CSK_4 (3). To confirm this finding, the expression of TLR2 was reduced in U937 macrophages with TLR2-targeting shRNA (Figure 2.11). U937 macrophages stably transduced with either non-targeting, control shRNA or TLR2-targeting shRNA were stimulated with Pam_3CSK_4 under serum-free conditions. shRNA targeting TLR2 reduced the IL-6 response to Pam_3CSK_4 by 73%, bringing the IL-6 response down to the level of unstimulated cells ($p = 0.037$) (Figure 3.1). These data confirm that TLR2 is necessary for the response to Pam_3CSK_4, and also demonstrate the efficacy of the shRNA-mediated knock down of TLR2 in U937 macrophages.

Figure 3.1

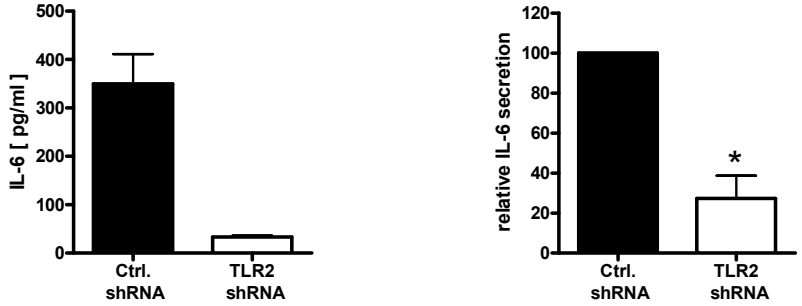


Figure 3.1 TLR2 mediates the U937 macrophage response to Pam_3CSK_4

U937 cells stably transduced with TLR2-specific shRNA (TLR2 shRNA) or non-targeting shRNA (Ctrl. shRNA) were stimulated with 100 ng/ml Pam_3CSK_4 for 6 hours under serum-free conditions. IL-6 was measured by ELISA. On the left are the data from one representative experiment showing mean IL-6 secretion (error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative IL-6 secretion compared to cells transduced with control shRNA (arbitrarily set to 100%, error bars represent the S.E.M.). Cells transduced with control shRNA secreted a mean of 200 pg/ml, and cells transduced with TLR2-targeting shRNA secreted a mean of 44 pg/ml.

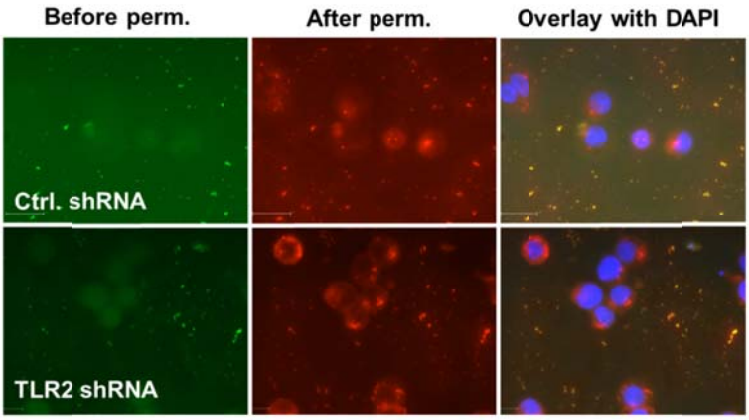
* $p = 0.037$.

TLR2 is not required for the endocytosis of Pam_3CSK_4

Whether TLRs participate in the endocytosis of their ligands seems to depend upon the TLR-ligand combination in question. While some studies demonstrate that TLR4 and TLR5 may be necessary for the efficient endocytosis of LPS and flagellin, respectively (128, 182), other studies have shown that TLR2 is not necessary for the endocytosis of TLR2/6 ligands (183). To determine whether TLR2 signaling participates in the endocytosis of Pam_3CSK_4, U937 macrophages stably transduced with either non-targeting, control shRNA or TLR2-targeting shRNA were incubated with Pam_3CSK_4-biotin for 60 minutes, then fixed and stained with anti-biotin antibodies before (FITC-labeled) and after (Texas Red-labeled) permeabilization (Figure 3.2A). The percent endocytosis was determined by counting the subset of macrophages with which Pam_3CSK_4-biotin molecules was associated, and determining the fraction of these macrophages that had internalized the ligand. Knockdown of TLR2 expression had no significant effect on the endocytosis of Pam_3CSK_4-biotin (Figure 3.2B). This data suggests that TLR2 signaling is not important in the endocytosis of Pam_3CSK_4.

Figure 3.2

A.


Before perm.
After perm.
Overlay with DAPI
Ctrl. shRNA
TLR2 shRNA

B.

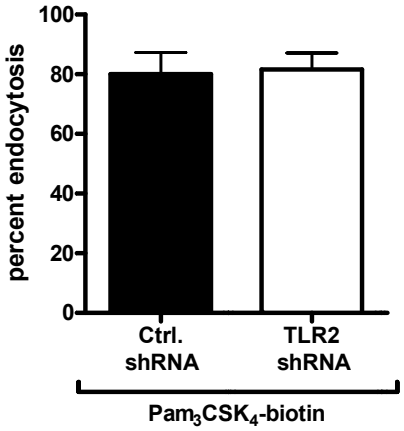

percent endocytosis
100
80
60
40
20
0
Ctrl. shRNA
TLR2 shRNA
Pam3CSK4-biotin

Figure 3.2 TLR2 signaling is not required for endocytosis of Pam_3CSK_4

(A) U937 cells were stably transduced with TLR2-specific shRNA (TLR2 shRNA) or non-targeting shRNA (Ctrl. shRNA) and stimulated with 5 μg/ml Pam_3CSK_4-biotin for 60 minutes. The cells were fixed and stained for immunofluorescent microscopy using α-biotin antibodies before (FITC) or after (Texas Red) permeabilization of the cells. Scale bars, 10μm. Data are representative of two independent experiments. **(B)** The endocytosis of Pam_3CSK_4-biotin was quantified by determining the percent endocytosis (the number of cells with internalized Pam_3CSK_4-biotin divided by number of cells with Pam_3CSK_4-biotin associated). Shown are the combined data from two independent experiments showing mean percent endocytosis (error bars represent S.E.M.). 4',6-diamidino-2-phenylindole (DAPI).

HEK293 cells express integrin $\alpha_3\beta_1$, and the inflammatory response to Pam_3CSK_4 in these cells is dependent upon integrin $\alpha_3\beta_1$ expression (Figure 1.13B). Since HEK293 cells (HEK) do not express TLR2, these cells present an opportunity to study the response to Pam_3CSK_4 in human cells that are completely deficient in TLR2 (as opposed to shRNA-transduced U937 macrophages in which some expression of TLR2 remains). HEK cells stably transfected with a TLR2-expression plasmid (HEK.TLR2) express significantly higher levels of TLR2 mRNA ($p = 0.037$) (Figure 3.3). To confirm the findings shown in Figure 3.2, HEK cells and HEK.TLR2 cells were incubated with Pam_3CSK_4-rhodamine for 60 minutes. The percentage of cells which had endocytosed the ligand was determined by Amnis Image Stream. Analysis of endocytosis by Amnis Image Stream is more quantitative than the counting methods traditionally employed by our laboratory. This software allows for the quantification of the endocytosis of Pam_3CSK_4-rhodamine by excluding plasma membrane-associate fluorescence; only internalized Pam_3CSK_4-rhodamine is considered in the analysis. No significant difference was observed in the endocytosis of Pam_3CSK_4-rhodamine in HEK.TLR2 cells compared with HEK cells (Figure 3.4B and C). Taken together, these data demonstrate that TLR2 signaling is not necessary for the efficient endocytosis of Pam_3CSK_4.

Figure 3.3

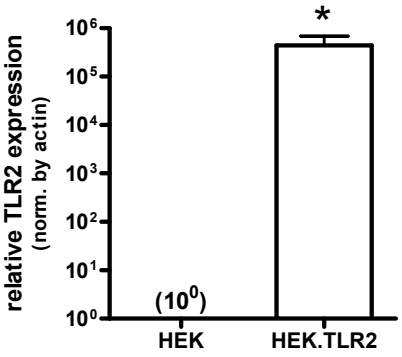


Figure 3.3 Expression of TLR2 mRNA in HEK293 and HEK.TLR2 cells

HEK293 cells were stably transfected with a TLR2-expressing plasmid (HEK.TLR2) or control (HEK) and analyzed by qRT-PCR. Shown are the combined data from three independent experiments showing mean TLR2 expression relative to control HEK293 cells (arbitrarily set to 1, error bars represent S.E.M.). * $p = 0.037$. Normalized (norm.).

Figure 3.4

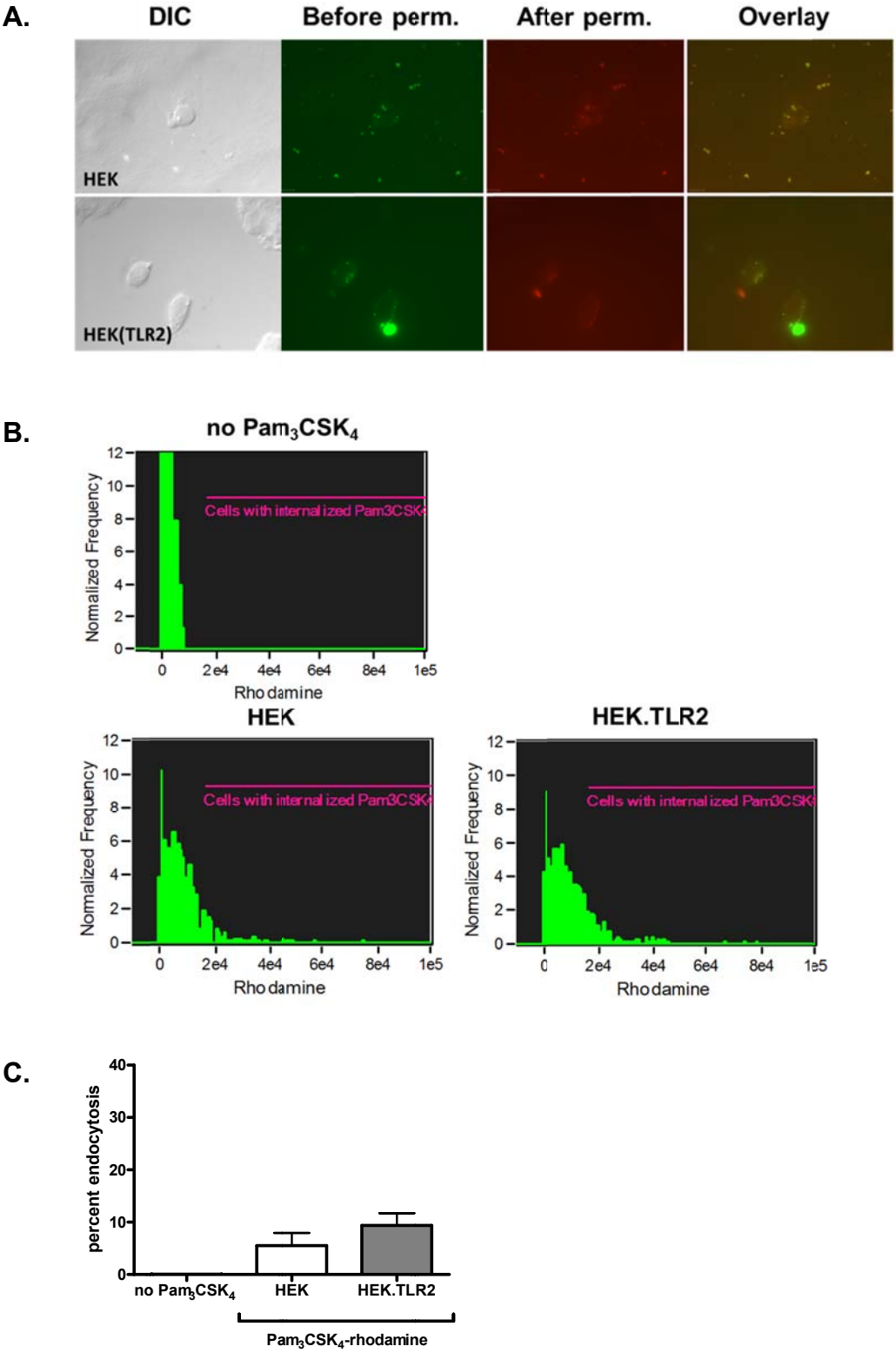

A.
DIC
Before perm.
After perm.
Overlay
HEK
HEK(TLR2)
B.
no Pam3CSK4
HEK
HEK.TLR2
Normalized Frequency
Rhodamine
Cells with internalized Pam3CSK
C.
percent endocytosis
no Pam3CSK4
HEK
HEK.TLR2
Pam3CSK4-rhodamine

Figure 3.4 HEK.TLR2 cells do not exhibit increased endocytosis

(A) HEK293 cells stably transfected with a TLR2-expressing plasmid (HEK.TLR2) or control (HEK), were stimulated with 5 μg/ml Pam_3CSK_4-biotin for 60 minutes. The cells were fixed and stained for immunofluorescent microscopy using α-biotin antibodies before (FITC) or after (Texas Red) permeabilization of the cells. Scale bars, 10μm. Data are representative of one independent experiment. **(B)** HEK293 cells stably transfected with a TLR2-expressing plasmid (HEK.TLR2) or control (HEK), were stimulated with 5 μg/ml Pam_3CSK_4-rhodamine for 60 minutes, and analyzed by Amnis Image Stream. Histograms are representative of three independent experiments. **(C)** The endocytosis of Pam_3CSK_4-rhodamine was quantified by determining the percent endocytosis (the number of cells with internalized Pam_3CSK_4-rhodamine as determined by Amnis Image Stream). Shown are the combined data from three independent experiments showing mean percent endocytosis (error bars represent S.E.M.). Differential interface contrast (DIC),

CD14 participates in the endocytosis of Pam_3CSK_4

Although CD14 is a known co-receptor for TLR2/1, the mechanism by which CD14 cooperates with TLR2/1 has not been elucidated. A previous study suggested that CD14 mediates endocytosis of TLR2/6 ligands, but whether this endocytosis was necessary for the inflammatory response to the ligand was not determined (183). We hypothesized that CD14 may also, with integrin $\alpha_3\beta_1$, mediate endocytosis of Pam_3CSK_4, and facilitate the recognition of Pam_3CSK_4 by TLR2/1 from endosomal compartments. To determine whether CD14 participates in the endocytosis of Pam_3CSK_4, we again examined endocytosis of Pam_3CSK_4 in HEK293 cells. While HEK cells (HEK) do not express high levels of CD14, HEK293 cells stably transfected with a CD14-expressing plasmid (HEK.CD14) express significantly higher levels of CD14 mRNA ($p = 0.037$) (Figure 3.5). HEK cells and HEK.CD14 cells were incubated with Pam_3CSK_4-rhodamine for 60 minutes. The percent endocytosis was determined by Amnis Image Stream. The percent endocytosis in HEK.CD14 cells was significantly higher than in HEK cells ($p = 0.05$) (Figure 3.6B and C). Taken together, these data demonstrate that CD14, along with integrin $\alpha_3\beta_1$, may play an important role in the efficient endocytosis of Pam_3CSK_4.

Figure 3.5

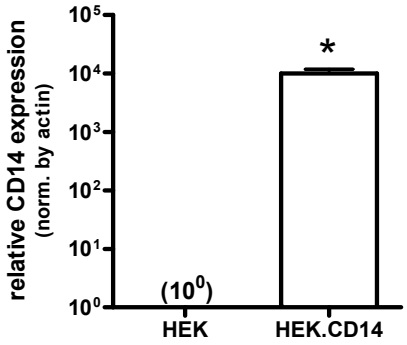


Figure 3.5 Expression of CD14 mRNA in HEK293 and HEK.CD14 cells

HEK293 cells were stably transfected with a CD14-expressing plasmid (HEK.CD14) or control (HEK) and analyzed by qRT-PCR. Shown are the combined data from three independent experiments showing mean CD14 expression relative to control HEK293 cells (arbitrarily set to 1, error bars represent S.E.M.). * $p = 0.037$. Normalized (norm.).

Figure 3.6

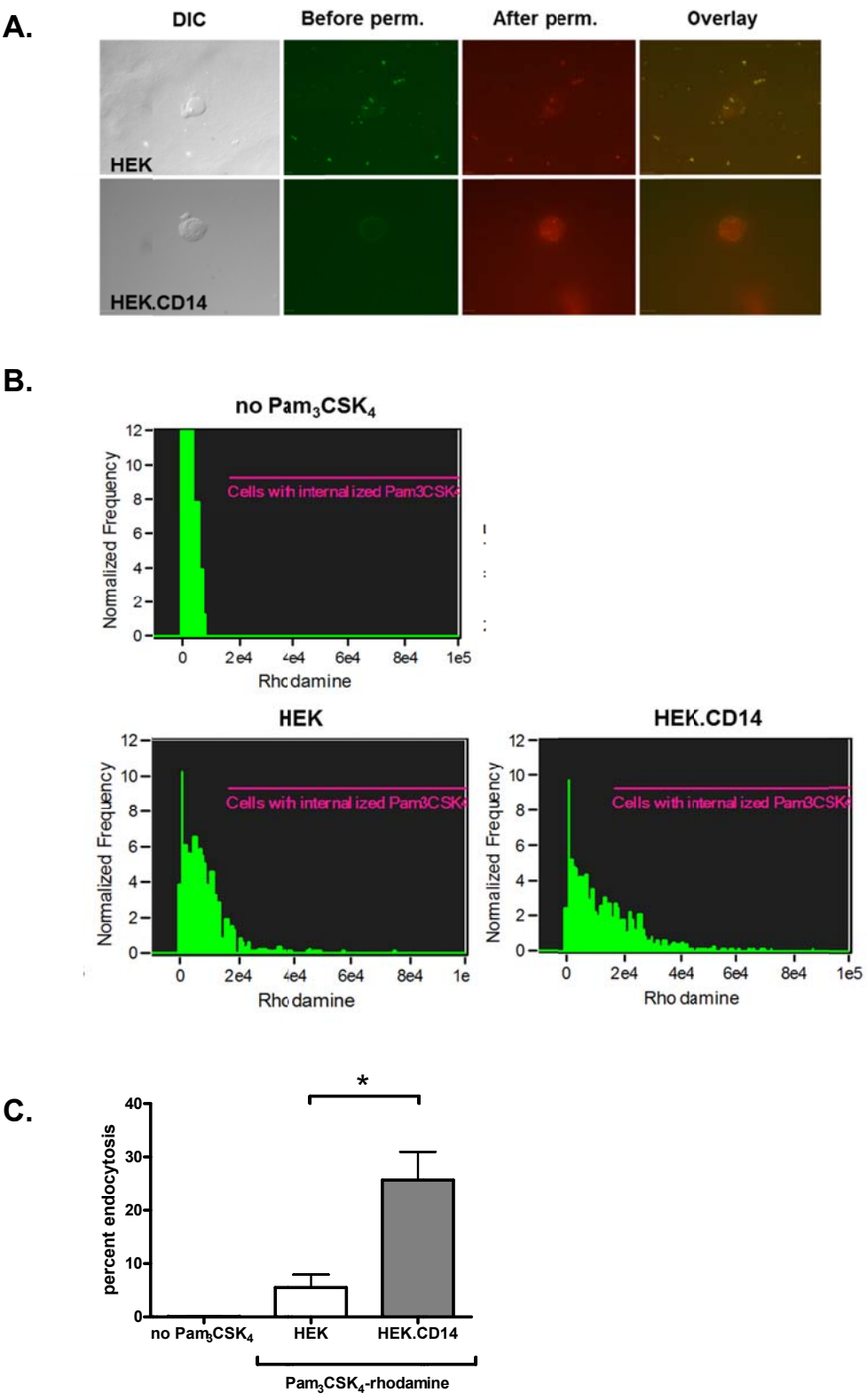

A.
DIC
Before perm.
After perm.
Overlay
HEK
HEK.CD14
B.
no Pam3CSK4
Normalized Frequency
Cells with internalized Pam3CSK4
Rhodamine
HEK
HEK.CD14
C.
percent endocytosis
no Pam3CSK4
HEK
HEK.CD14
Pam3CSK4-rhodamine
*

Figure 3.6 HEK.CD14 cells exhibit increased endocytosis of Pam_3CSK_4

(A) HEK293 cells stably transfected with a CD14-expressing plasmid (HEK.CD14) or control (HEK), were stimulated with 5 μg/ml Pam_3CSK_4-biotin for 60 minutes. The cells were fixed and stained for immunofluorescent microscopy using α-biotin antibodies before (FITC) or after (Texas Red) permeabilization of the cells. Scale bars, 10μm. Data are representative of one independent experiment. **(B)** HEK293 cells stably transfected with a CD14-expressing plasmid (HEK.CD14) or control (HEK), were stimulated with 5 μg/ml Pam_3CSK_4-rhodamine for 60 minutes, and analyzed by Amnis Image Stream. Histograms are representative of three independent experiments. **(C)** The endocytosis of Pam_3CSK_4-rhodamine was quantified by determining the percent endocytosis (the number of cells with internalized Pam_3CSK_4-rhodamine as determined by Amnis Image Stream). Shown are the combined data from three independent experiments showing mean percent endocytosis (error bars represent S.E.M.). * $p = 0.05$. Differential interface contrast (DIC).

CHAPTER 4

THE ROLE OF ADRENOMEDULLIN IN LYME DISEASE

Note: The data in this chapter has been published.

Marre ML, Darcy CT, Yinh J, Akira S, Uematsu S, Steere AC, Hu LT. 2010. Role of adrenomedullin in Lyme disease. *Infect Immun*. 78(12): 5307-5313.

CHAPTER 4 ABSTRACT

Borrelia burgdorferi stimulates a strong inflammatory response during infection of a mammalian host. To understand the mechanisms of immune regulation employed by the host to control this inflammatory response, we focused our studies on adrenomedullin, a peptide produced in response to bacterial stimuli that regulates inflammatory responses by modulating the expression of inflammatory cytokines. Specifically, we investigated the effect of *B. burgdorferi* on the expression of adrenomedullin as well as the ability of adrenomedullin to dampen host inflammatory responses to the spirochete. The concentration of adrenomedullin in the synovial fluid of untreated Lyme arthritis patients was elevated compared with control osteoarthritis patient samples. In addition, co-culture with *B. burgdorferi* significantly increased the expression of adrenomedullin in RAW264.7 macrophages through MyD88-, PI3-K-, and p38-dependent signaling cascades. Furthermore, the addition of exogenous adrenomedullin to *B. burgdorferi*-stimulated RAW264.7 macrophages resulted in a significant decrease in the induction of pro-inflammatory cytokines. Taken together, these results suggest that *B. burgdorferi* increases the production of adrenomedullin, which in turn negatively regulates the *B. burgdorferi*-stimulated inflammatory response.

CHAPTER 4 RESULTS

Adrenomedullin concentrations are elevated in the synovial fluid of patients with untreated Lyme arthritis

Previous reports have demonstrated that adrenomedullin is produced in response to inflammatory stimuli (87, 90, 123, 210). Because Lyme arthritis is an inflammatory arthritis, we sought to determine the concentration of adrenomedullin in the synovial fluid of Lyme arthritis patients. Synovial fluid was collected from the joints of patients presenting with either untreated Lyme arthritis or the non-inflammatory osteoarthritis. The use of a non-inflammatory form of arthritis as the negative control was necessary because the volume of synovial fluid in healthy joints is too small to be collected in sufficient quantities. The concentration of adrenomedullin in these synovial fluid samples was measured by radioimmunoassay. The median concentration of adrenomedullin in Lyme arthritis synovial fluids (0.22 pmol/ml) was 1.7-fold higher ($p = 0.005$) than that in osteoarthritis synovial fluids (0.13 pmol/ml) (Figure 4.1). Of note, the concentration of adrenomedullin in Lyme arthritis samples are significantly different from that in osteoarthritis samples, even when the two highest Lyme arthritis points are not considered ($p = 0.012$). As a second point of reference, the concentration of adrenomedullin was measured in samples from rheumatoid arthritis patients, because rheumatoid arthritis is characterized by a more inflammatory phenotype than Lyme arthritis. The median concentration of adrenomedullin in Lyme arthritis synovial fluids was 2.6-fold lower ($p = 0.005$) than that in rheumatoid arthritis synovial fluids (0.58 pmol/ml). Our data are

consistent with previous reports suggesting that adrenomedullin production correlates with levels of inflammation, and also demonstrate that adrenomedullin production is induced during Lyme arthritis.

Figure 4.1

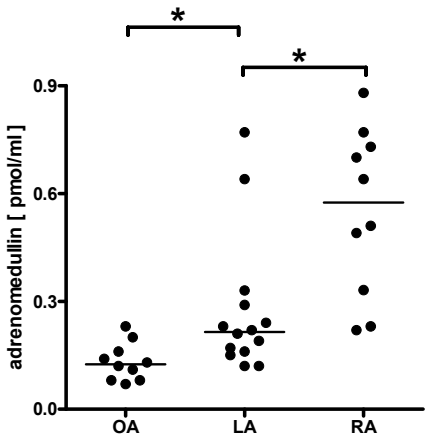


Figure 4.1 Adrenomedullin concentrations are elevated in the synovial fluid of patients with untreated Lyme arthritis

Synovial fluid was collected from patients with osteoarthritis (OA), untreated Lyme arthritis (LA), or rheumatoid arthritis (RA). The concentration of adrenomedullin in these samples was measured by radioimmunoassay. Horizontal bars represent the median of each group. * p = 0.005.

B. burgdorferi co-culture induces adrenomedullin expression in RAW264.7 macrophages

In addition to inflammatory stimuli, adrenomedullin expression can be induced by bacteria and bacterial products (2, 124, 223, 228). To determine whether *B. burgdorferi* can induce the expression of adrenomedullin in host cells, RAW264.7 macrophages were co-cultured with *B. burgdorferi* at varying multiplicities of infection (MOI 0.1, 1, 10, or 100), and adrenomedullin expression was measured by qRT-PCR. Adrenomedullin expression was significantly induced in a dose-dependent manner after 24 hours of co-culture ($p = 0.037$) (Figure 4.2).

Figure 4.2

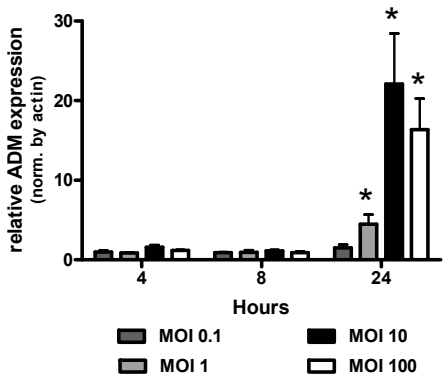


Figure 4.2 *B. burgdorferi* co-culture induces adrenomedullin expression in RAW264.7 macrophages

RAW264.7 cells were co-cultured with *B. burgdorferi* for 4, 8, or 24 hours. Adrenomedullin (ADM) expression was measured by qRT-PCR. Shown are the combined data from three independent experiments showing mean adrenomedullin expression relative to uninfected cells (arbitrarily set to 1 (not shown), error bars represent S.E.M.). * $p = 0.037$. Normalized (norm.).

The induction of adrenomedullin by *B. burgdorferi* requires signaling through MyD88, PI3-K, and p38

We next sought to identify which signaling molecules regulate adrenomedullin expression in our experimental system. We and others have previously shown that *B. burgdorferi* activates a number of signaling pathways, including TLR2-MyD88 signaling, activation of the MAPK p38, and activation of phosphatidylinositol 3-kinase (PI3-K) (7, 19, 86, 104, 188, 189, 215, 216). We therefore examined the potential roles for these molecules in the induction of adrenomedullin by *B. burgdorferi*.

To determine whether MyD88 is important for the regulation of adrenomedullin in response to *B. burgdorferi*, wild type or MyD88$^{-/-}$ bone marrow-derived macrophages were co-cultured with *B. burgdorferi* at MOI 10 for 24 hours. Adrenomedullin expression was measured by qRT-PCR. Deficiency in MyD88 resulted in an 83% decrease in adrenomedullin expression compared to wild type cells ($p = 0.014$) (Figure 4.3A).

We next examined the potential roles in the induction of adrenomedullin for signaling molecules which are activated downstream of MyD88 in response to *B. burgdorferi*. RAW264.7 macrophages were pre-treated with LY294002 (10 μM), which inhibits the activity of PI3-K and has been shown to inhibit the phagocytosis of *B. burgdorferi* (189), or with SB203580 (3 μM), which inhibits the activity of p38 MAPK and the inflammatory response to *B. burgdorferi* (7, 19),

prior to the addition of *B. burgdorferi* at MOI 10 for 24 hours. Pretreatment with LY294002 or SB203580 significantly inhibited the induction of adrenomedullin by 79% and 57%, respectively ($p = 0.037$) (Figure 4.3B). Taken together, these data suggest that, in response to *B. burgdorferi*, transduction of inflammatory signals through MyD88, PI3-K, and p38 MAPK is necessary for the up-regulation of adrenomedullin expression.

Figure 4.3

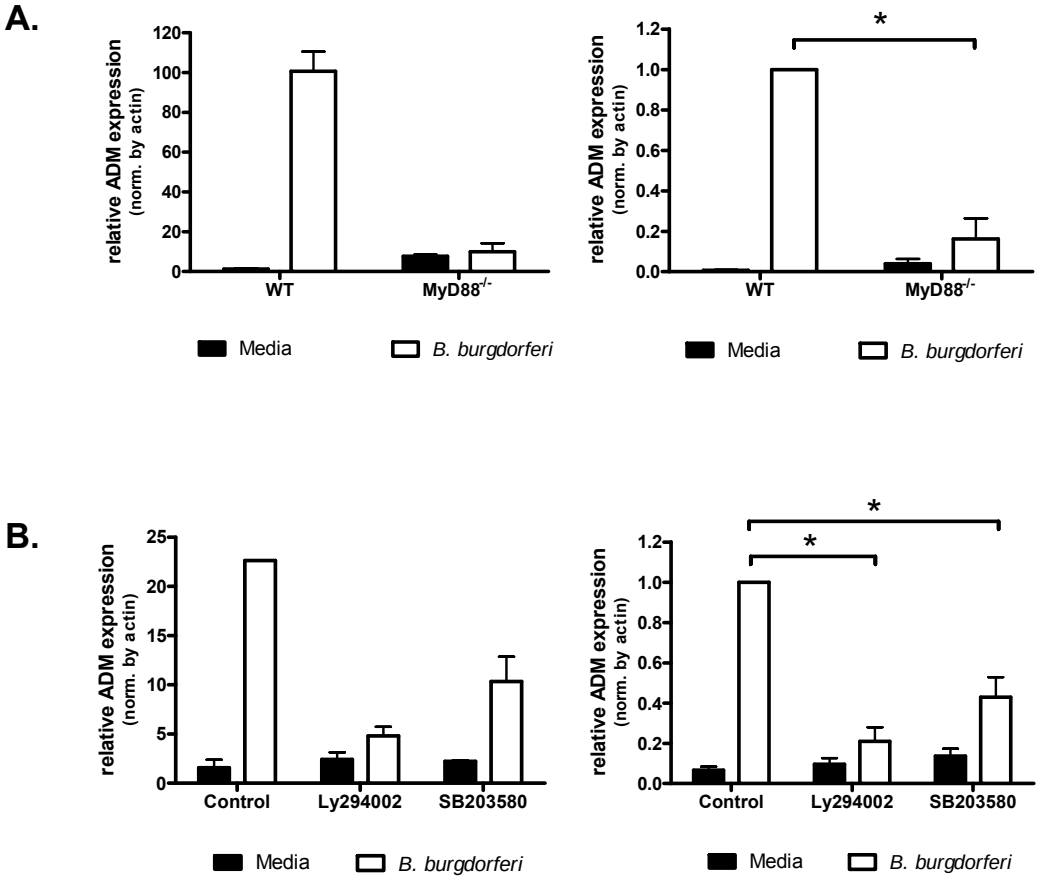

A.
relative ADM expression
(norm. by actin)
120
100
80
60
40
20
0
WT
MyD88-/-
Media
B. burgdorferi
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
B.
25
20
15
10
5
Control
Ly294002
SB203580

Figure 4.3 The induction of adrenomedullin by *B. burgdorferi* requires signaling through MyD88, PI3-K, and p38

(A) Wild type (WT) or MyD88-defiicient ($MyD88^{-/-}$) bone marrow-derived macrophages were co-cultured with *B. burgdorferi* at MOI 10 for 24 hours. Adrenomedullin (ADM) expression was measured by qRT-PCR. On the left are the data from one representative experiment showing mean adrenomedullin expression compared to wild type macrophages incubated with media alone (arbitrarily set to 1, error bars represent S.D. of duplicates). On the right are the combined data from four independent experiments showing relative adrenomedullin expression compared to wild type macrophages co-cultured with *B. burgdorferi* (arbitrarily set to 1, error bars represent the S.E.M.). * p = 0.014. **(B)** RAW264.7 cells were pre-treated with Ly294002 (10 μM), SB203580 (3 μM), or control, for 2 hours prior to co-culture with *B. burgdorferi* MOI 10 for 24 hours. Adrenomedullin (ADM) expression was measured by qRT-PCR. On the left are the data from one representative experiment showing mean adrenomedullin expression compared to control cells incubated with media alone (arbitrarily set to 1, error bars represent S.D. of duplicates). On the right are the combined data from three independent experiments showing relative adrenomedullin expression compared to control cells co-cultured with *B. burgdorferi* (arbitrarily set to 1, error bars represent the S.E.M.). * p = 0.037. Normalized (norm.).

The induction of adrenomedullin by *B. burgdorferi* requires new protein synthesis

Previous publications have demonstrated that adrenomedullin mRNA is induced in response to bacterial ligands as early as 2 hours post stimulation (124, 228). The observation that *B. burgdorferi* co-culture induces adrenomedullin mRNA only at 24 hours post infection (Figure 4.2) suggested that this induction may require synthesis of intermediary proteins rather than direct induction by recognition of *B. burgdorferi*. To determine whether intermediate protein synthesis is necessary for the induction of adrenomedullin mRNA, RAW264.7 macrophages were pre-treated with cycloheximide (5 μM), which inhibits mRNA translation on cytosolic 80S ribosomes, or control for 2 hours prior to the addition of *B. burgdorferi* at MOI 10 for 24 hours. Pretreatment with cycloheximide reduced the induction of adrenomedullin mRNA in response to co-culture with *B. burgdorferi* by 88% ($p = 0.013$) (Figure 4.4). These results support the hypothesis that the inflammatory environment induced by *B. burgdorferi*, rather than *B. burgdorferi* itself, stimulates adrenomedullin expression.

Figure 4.4

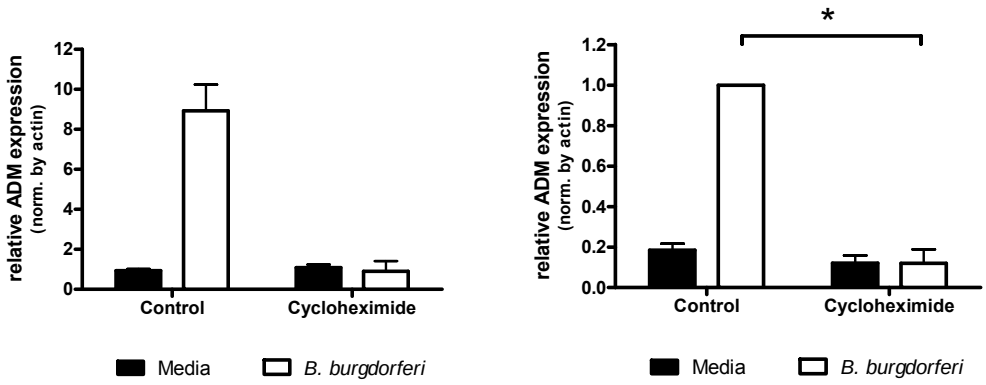


Figure 4.4 The induction of adrenomedullin by *B. burgdorferi* requires new protein synthesis

RAW264.7 cells were co-cultured with *B. burgdorferi* at MOI 10 for 24 hours in the presence of cycloheximide (5 μM) or control. Adrenomedullin (ADM) expression was measured by qRT-PCR. On the left are the data from one representative experiment showing mean adrenomedullin expression compared to control cells incubated with media alone (arbitrarily set to 1, error bars represent S.D. of duplicates). On the right are the combined data from four independent experiments showing relative adrenomedullin expression compared to control cells co-cultured with *B. burgdorferi* (arbitrarily set to 1, error bars represent the S.E.M.). * p = 0.013. Normalized (norm.).

Adrenomedullin decreases the *B. burgdorferi*-induced production of pro-inflammatory cytokines

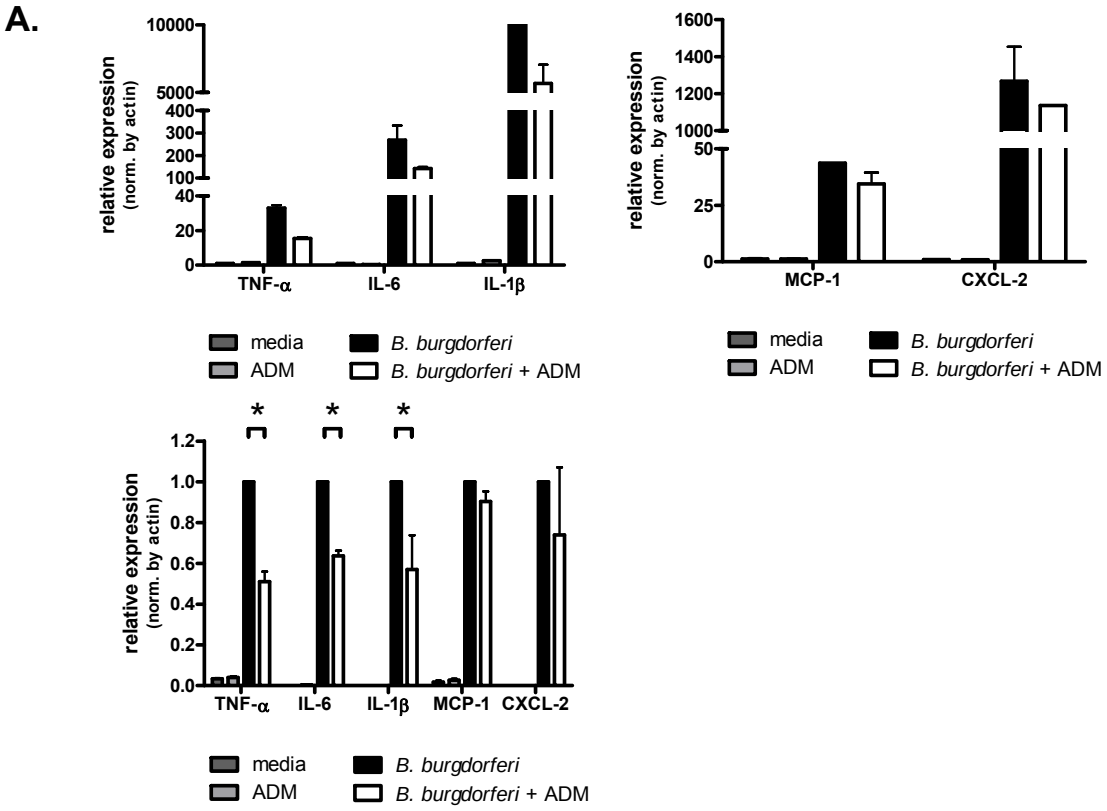
Adrenomedullin regulates inflammatory responses through the ligation of its receptor and the initiation of signaling cascades which down-regulate the expression of pro-inflammatory mediators (102, 112, 147, 218). However, the immunomodulatory properties of adrenomedullin have never been examined in the context of an inflammatory response stimulated by a whole bacterium. We therefore sought to determine whether adrenomedullin can regulate the expression of inflammatory cytokines induced by *B. burgdorferi*.

RAW264.7 macrophages were co-cultured with *B. burgdorferi* at MOI 1 for 4 hours in the presence or absence of 100 nM adrenomedullin. This early time point is well before the time at which RAW264.7 macrophages begin expressing adrenomedullin in response to *B. burgdorferi* (Figure 4.2). Thus, any effect of adrenomedullin can be attributed to the exogenous adrenomedullin added at the time of stimulation. The addition of adrenomedullin to *B. burgdorferi*-RAW264.7 macrophage co-cultures did not decrease the expression of chemokines MCP-1 and CXCL-2, but did significantly decrease the expression of mRNA for inflammatory cytokines TNF-α (49%), IL-6 (36%), and IL-1β (43%) ($p = 0.037$) (Figure 4.5A).

To confirm this anti-inflammatory role of adrenomedullin, the concentrations of TNF-α and IL-6 in the supernatants of RAW264.7 macrophage cell cultures were

measured by ELISA. IL-1β is not secreted by RAW264.7 macrophages in response to *B. burgdorferi* (data not shown). RAW264.7 macrophages were co-cultured with *B. burgdorferi* at MOI 10 for 6 hours in the presence or absence of 100 nM adrenomedullin. In this experiment, a higher MOI and later time point were used to induce detectable levels of cytokine secretion. Similar to observations made using qRT-PCR, the addition of adrenomedullin to co-cultures of RAW264.7 macrophages and *B. burgdorferi* significantly decreased the secretion of TNF-α and IL-6 by 21% and 43%, respectively (Figure 4.5B). These data demonstrate that adrenomedullin is capable of regulating the host response to *B. burgdorferi* by reducing the induction of pro-inflammatory cytokines in macrophages.

Figure 4.5


A.
relative expression
(norm. by actin)
TNF-α
IL-6
IL-1β
MCP-1
CXCL-2
media
ADM
B. burgdorferi
B. burgdorferi + ADM
*

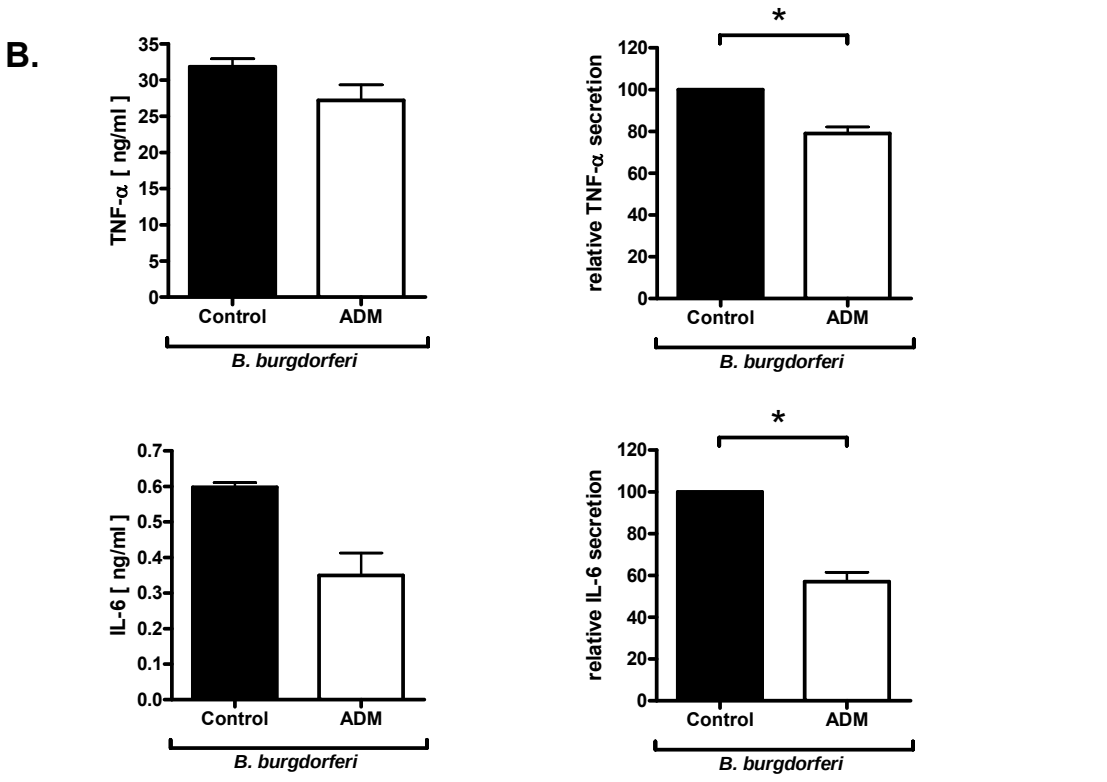

B.
TNF-α [ng/ml]
relative TNF-α secretion
IL-6 [ng/ml]
relative IL-6 secretion
Control
ADM
B. burgdorferi
*

Figure 4.5 Adrenomedullin decreases the *B. burgdorferi*-induced production of pro-inflammatory cytokines

(A) RAW264.7 cells were co-cultured with *B. burgdorferi* MOI 1 for 4 hours in the presence of 100 nM adrenomedullin (ADM) or control. Expression of inflammatory cytokines and chemokines was measured by qRT-PCR. On the top left are the data from one representative experiment showing mean cytokine expression compared to cells incubated with media alone (arbitrarily set to 1, error bars represent S.D. of duplicates). On the top right are the data from one representative experiment showing mean chemokine expression compared to cells incubated with media alone (arbitrarily set to 1, error bars represent S.D. of duplicates). On the bottom are the combined data from three independent experiments showing relative cytokine or chemokine expression compared to cells co-cultured with *B. burgdorferi* (arbitrarily set to 1, error bars represent the S.E.M.). * $p = 0.037$. Normalized (norm.) **(B)** RAW264.7 cells were co-cultured with *B. burgdorferi* MOI 10 for 6 hours in the presence of 100 nM adrenomedullin (ADM) or control. Secretion of TNF-α and IL-6 were measured by ELISA. On the top left are the data from one representative experiment showing mean TNF-α secretion (error bars represent S.D. of triplicates). On the top right are the combined data from four independent experiments showing relative TNF-α secretion compared to control cells (arbitrarily set to 100%, error bars represent the S.E.M.). Control cells secreted a mean of 29.16 ng/ml, and adrenomedullin-treated cells secreted a mean of 23.028 ng/ml. On the bottom left are the data from one representative experiment showing mean IL-6 secretion (error bars

represent S.D. of triplicates). On the top right are the combined data from four independent experiments showing relative IL-6 secretion compared to control cells (arbitrarily set to 100%, error bars represent the S.E.M.). Control cells secreted a mean of 0.825 ng/ml, and adrenomedullin-treated cells secreted a mean of 0.470 ng/ml. * $p = 0.014$.

CHAPTER 5

DISCUSSION

The inflammatory response of macrophages to bacteria involves the engagement of many different receptors both on the plasma membrane and in sub-cellular compartments. The mechanisms by which these different receptors interact to mediate inflammation are only beginning to be understood. Here, in Chapters 2 and 3, we have demonstrated four important findings. First, integrin $\alpha_3\beta_1$ cooperates with TLR2/1 to facilitate inflammatory responses to bacterial lipopeptides. Second, the mechanism by which integrin $\alpha_3\beta_1$ cooperates with TLR2/1 responses is through the role of integrin $\alpha_3\beta_1$ in mediating the endocytosis of Pam_3CSK_4 and *B. burgdorferi*, thus facilitating the recognition of ligands by TLR2/1 within the endosome. Third, TLR2/1 signals from endosomal compartments results in the induction of a different subset of inflammatory mediators than does TLR2 signaling from the plasma membrane. Fourth, we have determined the roles of TLR2 and CD14 in the endocytosis of and response to Pam_3CSK_4. Specifically, although TLR2 is internalized and recognizes Pam_3CSK_4 from endosomal compartments, TLR2 itself is not required for the endocytosis of Pam_3CSK_4, while CD14, a known co-receptor for TLR2, enhances the endocytosis of Pam_3CSK_4.

Our current model for the role of integrin $\alpha_3\beta_1$ and CD14 in facilitating TLR2/1 signaling is shown in Figure 5.1. Because binding of either Pam_3CSK_4 or *B. burgdorferi* to cells is independent of integrin $\alpha_3\beta_1$, we hypothesize that lipoproteins attach to the cell through interaction with integrin $\alpha_V\beta_3$ and/or CD14. This attachment brings the ligand into proximity with TLR2/1 at the plasma

membrane, facilitating TLR2/1 signaling through its canonical signaling pathways which result in early NF-κB activation and the induction of inflammatory cytokines such as TNF-α. In addition, integrin $\alpha_3\beta_1$, in collaboration with CD14, facilitates endocytosis of Pam_3CSK_4 or *B. burgdorferi*, perhaps together with TLR2/1. Once localized within the endosome, TLR2/1 recognizes its ligand and recruits adaptor molecules such as MyD88 (or another adaptor such as TRIF) to activate signaling pathways which result in late NF-κB activation and induction of inflammatory cytokines such as IL-6, as well as the activation of IRFs and the induction of IFN-I such as IFN-α1.

Figure 5.1

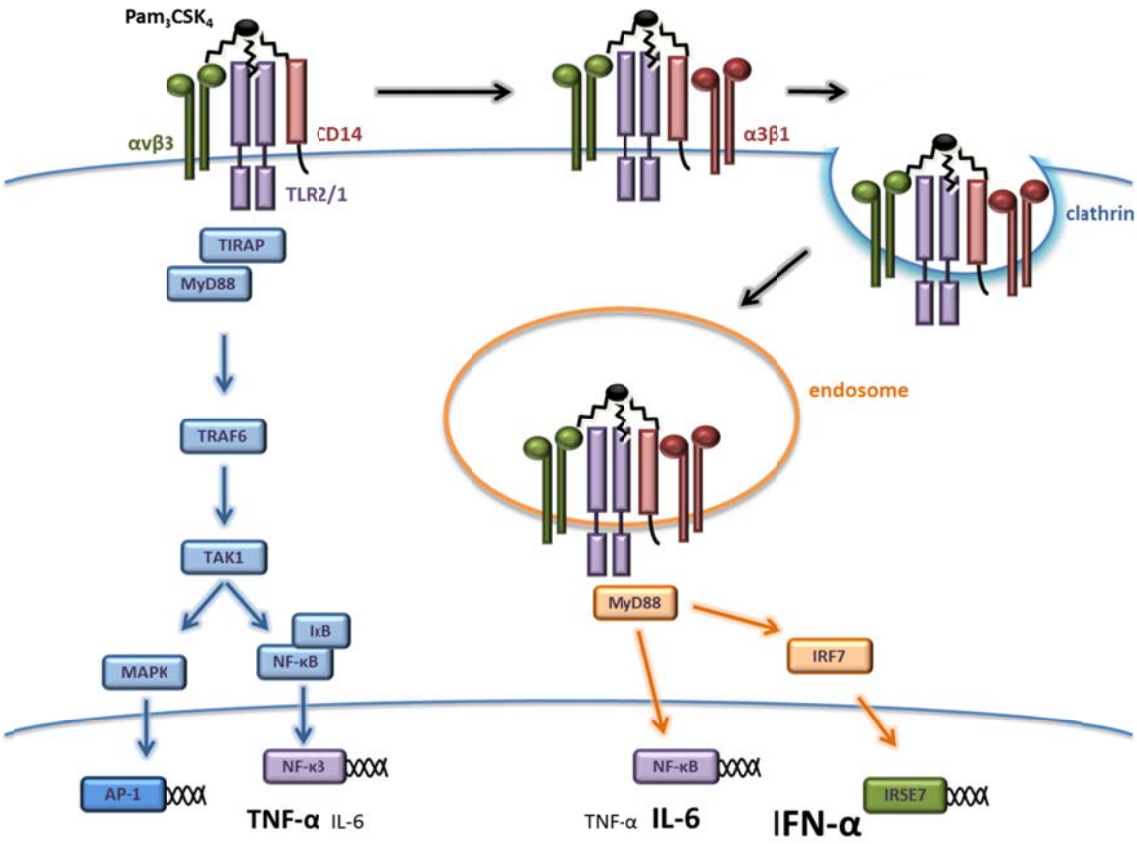


Figure 5.1 Model of TLR2/1-mediated responses to bacterial lipopeptides

Pam_3CSK_4 attaches to the surface of macrophages through interaction with integrin $\alpha_V\beta_3$ and/or CD14. At the surface of macrophages, Pam_3CSK_4 interacts with TLR2/1 heterodimers, activating the canonical TLR2/1 signaling cascade. In addition, through the co-operation of integrin $\alpha_3\beta_1$ and CD14, Pam_3CSK_4 is endocytosed through clathrin-dependent mechanisms. In endosomal compartments, Pam_3CSK_4 again interacts with TLR2/1 heterodimers, initiating different signaling cascades and inducing the expression of a different subset of inflammatory mediators.

Ours is not the first report proposing an integrin as a TLR co-receptor. Several mechanisms by which integrins cooperate with TLRs have now been described. For instance, several groups have demonstrated a role for integrin $\alpha_M\beta_2$ in the TLR4-dependent inflammatory response to LPS (98, 110, 166). Kagan et al. demonstrated that integrin $\alpha_M\beta_2$ facilitates TLR4 by regulating the generation of PIP_2 in the plasma membrane, which is necessary for the recruitment of MAL to the cytoplasmic tail of TLR4 to initiate signaling (110). More recently, Gerold et al. showed that integrin $\alpha_V\beta_3$ is necessary for the complete inflammatory response to many TLR2 ligands, including Pam_3CSK_4. This group suggested that the TLR2 ligands interact with vitronectin, the ECM ligand for integrin $\alpha_v\beta_3$. Then, when integrin $\alpha_v\beta_3$ binds its ligand, this ligation indirectly mediates the attachment of lipopeptides to the macrophages, which leads to clustering of the lipopeptide with TLR2/1 at the cell surface and facilitates signaling (65).

Previous work from our laboratory demonstrated a role for integrin $\alpha_3\beta_1$ in the inflammatory response to *B. burgdorferi*. However, studies in TLR-deficient HEK293 cells suggests that integrin $\alpha_3\beta_1$ may not induce an inflammatory response to *B. burgdorferi* in the absence of TLR expression (Figure 1.13). We therefore sought to determine whether integrin $\alpha_3\beta_1$ cooperates with TLRs, especially TLR2/1, in mediating the inflammatory response to *B. burgdorferi*. We show here that inhibition of integrin $\alpha_3\beta_1$ prevents the inflammatory response to Pam_3CSK_4. These data suggest that integrin $\alpha_3\beta_1$ cooperates with TLR2/1 to mediate the inflammatory response to bacterial lipopeptides.

Although ours is not the first report to propose an integrin as a co-receptor for a TLR, our data suggests a novel mechanism of cooperation between integrins and TLRs. Integrin $\alpha_v\beta_3$ was proposed to mediate attachment of bacterial lipopeptides to cell surfaces through binding of vitronectin attached to the lipopeptide (65). Our results differ in that the addition of exogenous serum (which contains vitronectin as well as ECM ligands for integrin $\alpha_3\beta_1$) does not affect the inflammatory response to Pam_3CSK_4 in U937 macrophages. In addition, since our experiments were performed in serum-free media, we have shown that the absence of exogenous serum does not affect the requirement for integrin $\alpha_3\beta_1$ in the cytokine response to bacterial lipopeptides. The fact that down-regulation of integrin α_3 does not decrease attachment of Pam_3CSK_4 to the macrophages further supports the case that integrin $\alpha_3\beta_1$ plays a different role than integrin $\alpha_v\beta_3$ in facilitating TLR2/1 signaling.

Although integrins are being increasingly recognized as important mediators of internalization of host factors as well as bacterial and viral ligands (52), this is the first report demonstrating that an integrin mediates the endocytosis of synthetic bacterial lipopeptides. Integrin-associated mechanisms of endocytosis include recruitment of clathrin, caveolin, and dynamin to the endocytic cup (36). These findings are consistent with our observations that chemical inhibition of clathrin also blocks IL-6 induction in response to Pam_3CSK_4.

Whether integrin $\alpha_3\beta_1$ plays a similar role in facilitating responses to live bacterial pathogens was determined by testing responses to *B. burgdorferi*, a bacterium characterized by its high expression of lipoproteins. We demonstrated that integrin $\alpha_3\beta_1$ is important in the endocytosis of this organism, facilitating the recognition of borrelial ligands from within endosomal compartments by receptors including TLR2/1. Although we cannot rule out the involvement of other endosomal receptors (primarily TLR7 (168); *B. burgdorferi* does not activate TLR4 (130) and U937 macrophages are unresponsive to TLR9 ligands (222)), our data show that TLR2/1 plays the major role in regulating the IL-6 response to *B. burgdorferi*.

Based on previous data demonstrating a role for integrin signaling in the initiation of endocytosis (52), we hypothesize that signaling pathways downstream of integrin $\alpha_3\beta_1$ must be activated to trigger the endocytosis of Pam_3CSK_4 and *B. burgdorferi*. However, although several integrins have been linked to endocytic processes (52), the signaling pathways that link the integrins to the formation of an endocytic cup for the internalization of small particles (such as Pam_3CSK_4) have not been defined. Therefore, it would be an important advancement for the understanding of integrin biology and endocytosis to map the signaling pathways activated by integrin $\alpha_3\beta_1$ for the endocytosis of Pam_3CSK_4 and *B. burgdorferi*.

Our data presented here clearly show that TLR2/1 heterodimers recognize Pam_3CSK_4 from endosomal compartments, and initiate inflammatory signaling

cascades from these compartments. There has been significant controversy regarding whether TLR2/1 is active within endosomal compartments. TLR2 is clearly recruited to endosomal membranes (211), but its ability to signal from these compartments remains controversial. It has been suggested that TLR2 can only signal from the plasma membrane because its adaptor MAL does not localize to intracellular compartments (110, 111). In this model, the MAL/MyD88 adaptor complex would dissociate from TLR2 prior to its inclusion in an endosomal membrane, leaving it unable to signal. However, other studies have shown that signaling defects caused by MAL deficiency can be overcome by higher levels of Pam_3CSK_4 stimulation (114) and MAL deficient mice are still capable of an inflammatory response to TLR2 ligands (47, 91) suggesting that the inability of MAL to traffic to endosomes does not exclude the possibility of TLR2 signaling from these compartments.

Recently, Barbalat et al. showed that TLR2 signals from endosomal compartments of specialized mouse inflammatory monocytes in response to virus, but not in response to Pam_3CSK_4 (11). However, a subsequent study by Dietrich et al. demonstrated that synthetic bacterial lipopeptides also induce TLR2-mediated signals from endosomal compartments of murine bone marrow-derived macrophages (51). Our data also show that bacterial lipopeptides are recognized from within endosomes by TLR2/1, providing strong evidence to resolve this issue. Subsequently, several additional studies have been published

which also support the hypothesis that TLR2 recognizes ligands in, and signals from, endosomal compartments (38, 99, 146).

While our study and the studies by Barbalat et al. and Dietrich et al. agree that TLR2 signals from endosomes, there are differences in the observed inflammatory response generated by endosomal TLR2/1. One study showed that TLR2 induces IFN-α and IFN-β induction in response to virus, but not in response to Pam_3CSK_4 (11). In contrast, another study showed that bacterial TLR2 ligands, including Pam_3CSK_4, stimulate the induction of IFN-β (51). We did not observe an induction of IFN-β in response to Pam_3CSK_4 in human U937 macrophages; however, this may be due to differences in cell type, since we did observe Pam_3CSK_4-induced IFN-β in murine bone marrow derived macrophages (data not shown). We also found that bacterial lipopeptide stimulation does indeed induce at least one subtype of IFN-α. We found that both *B. burgdorferi* (data not shown) and Pam_3CSK_4 induce mRNA for IFN-α1 in U937 macrophages, and that this induction could be almost completely inhibited by the addition of endosomal acidification inhibitors. The discrepancy between our data and previous reports suggesting that Pam_3CSK_4 does not induce IFN-α is likely due to the facts that different subtypes of IFN-α were measured in each of the different studies, and that each of these previous studies were performed in different cell types (11, 51, 141).

Our study also differs from previous claims that TLR2/1 does not induce pro-inflammatory cytokines from the endosome (11, 51, 111) as we show clear evidence that internalization of Pam_3CSK_4 and endosomal acidification is necessary for the complete IL-6 response. It has been suggested that the involvement of the IFN-β autocrine/paracrine loop enhances NF-κB-mediated induction of IL-6 (51). It is unclear how much IFN-β contributes to IL-6 production, as studies addressing this point in different cell types and downstream of different stimuli have produced variable results (58, 61, 64, 149, 206). In U937 macrophages, we observe no induction of IFN-β in response to Pam_3CSK_4, suggesting that IL-6 secretion in our system is not controlled by IFN-β signaling. Therefore, the decrease we observe in IL-6 production upon treatment with endosomal acidification inhibitors is likely due to the more classical endosomal TLR-mediated induction of IL-6 through NF-κB.

The importance of endocytosis and endosomal maturation in the response to a whole bacterium such as *B. burgdorferi* is thought to be due to the importance of processing and degradation of the bacteria within endosomal compartments (49, 99, 188). However, the importance of endosomal maturation in the response to synthetic Pam_3CSK_4 is not understood. One possibility is that synthetic lipids form micelles in aqueous solution which require processing by pH-dependent endosomal enzymes prior to recognition by TLR2/1. Alternatively, like TLR9, endosomal TLR2/1 may itself require processing to recognize Pam_3CSK_4 and transduce signals from the endosome. Finally, some evidence suggests that the

fusion of an endosome with a low pH lysosome is necessary for the recruitment of signaling molecules to these compartments (82, 138). These data would suggest that ligated TLR2/1 may not signal in early endosomes, but only in mature endolysosomes with a pH that allows recruitment of MyD88 and other signaling adaptors. Further work is necessary to understand the importance of endosomal maturation in the response to the synthetic ligand Pam_3CSK_4.

Sub-cellular localization of TLR2 has been suggested to generate specificity in the inflammatory response (11, 51). Although the induction of IL-6 by Pam_3CSK_4 was significantly decreased by endosomal acidification inhibitors, the same inhibitors had much less effect on TNF-α secretion by U937 macrophages (data not shown). This is consistent with the observations in murine macrophages (51). Conversely, we found that the effects of endosomal acidification inhibitors on IFN-α1 were more pronounced than the effects on IL-6, suggesting a greater dependence on endosomal signaling for IFN-α1. That different cytokines induced by Pam_3CSK_4 are affected differently by endosomal acidification inhibitors suggests that TLR2/1 responses are likely to be context dependent, in that, signaling from TLR2/1 localized to the plasma membrane may differ from signaling activated by TLR2/1 in endosomes. We propose that induction of TNF-α occurs primarily from plasma membrane TLR2/1, that induction of IFN-α1 occurs primarily from endosomal TLR2/1, and that IL-6 may be induced by both plasma membrane and endosomal TLR2/1. Similar observations were made in a macrophage model of the inflammatory response to *Staphylococcus aureus*

(99). The differences in cytokine profiles may be explained, in part, by the recruitment of different signaling adapters to TLR2/1 at the plasma membrane and TLR2/1 in the endosome. For instance, MAL is hypothesized to facilitate TLR signaling at the plasma membrane, but not at the endosome (110, 111). Conversely, IRFs are thought to be recruited to endosomal membranes, but not to the plasma membrane (14, 89, 111). The differences in cytokine profiles resulting from context-dependent TLR2 signaling, as well as the mechanisms by which cellular context alters TLR2/1 signaling, remain to be determined.

Although our data demonstrate an important role for integrin $\alpha_3\beta_1$ in the endocytosis of Pam_3CSK_4, shRNA targeting of integrin α_3 only results in a 46% decrease in the endocytosis of Pam_3CSK_4 (Figure 2.4C and D). This suggests that, although integrin $\alpha_3\beta_1$ contributes significantly to the endocytosis of Pam_3CSK_4, integrin $\alpha_3\beta_1$ may not be the only endocytic receptor for this ligand. We therefore sought to identify other TLR2 co-receptors which might co-operate with integrin $\alpha_3\beta_1$ in the internalization of Pam_3CSK_4.

One mechanism of cooperation between integrins and other receptors is inside-out signaling. Inside-out signaling cascades originate from other receptors in response to external stimuli, and activate integrins to increase their affinity for ligand (136). In some cases, this inside-out signaling is mediated by TLR2 (77-79). We therefore hypothesized that TLR2/1 signaling from the plasma membrane could aid in the activation of integrin $\alpha_3\beta_1$, thus indirectly participating

in the endocytosis of its ligand. However, U937 macrophages deficient in TLR2 do not exhibit decreased endocytosis of Pam_3CSK_4. In addition, there was no significant difference in the endocytic index between HEK293 cells, and HEK293 cells expressing TLR2. Thus, although we have not directly addressed whether TLR2/1 mediates inside-out signaling to activate integrin $\alpha_3\beta_1$, we have clearly demonstrated that TLR2 signaling in not necessary for the endocytosis of Pam_3CSK_4. These data are consistent with previous publications which have shown that TLR2 is neither necessary nor sufficient for the endocytosis of the TLR2/6 ligand FSL-1 (183).

CD14 is a well-known co-receptor for both TLR2 and TLR4 (3). As a co-receptor for TLR4, CD14 is absolutely required for the TLR4-mediated cellular response to LPS (3), as CD14 is crucial for the association of LPS with TLR4 on the plasma membrane. CD14 also participates in TLR2 signaling events, but is not absolutely required (139, 179, 225). That is, inflammatory responses to TLR2 ligands are impaired, but not absent, in CD14-deficient cells (153). The mechanism by which CD14 cooperates with TLR2/1 has not been well described. CD14 has been shown to serve as a tethering receptor in other systems (139, 192), so we hypothesize that CD14 may be important in the initial attachment of Pam_3CSK_4 to macrophages. In addition, we have now shown that, in HEK293 cells, over-expression of CD14 enhances to the endocytosis of Pam_3CSK_4, thus facilitating the recognition of Pam_3CSK_4 by endosomal TLR2/1. While this function of CD14 has also been proposed for TLR2/6-mediated recognition of

FSL-1 (183) and for TLR3-, TLR7-, and TLR9-mediated recognition of nucleic acids (15, 127), our data is the first to suggest that CD14 mediates endocytosis of TLR2/1 ligands. Further work will be necessary to determine whether CD14 also mediates endocytosis of Pam_3CSK_4 in macrophages, and to elucidate the mechanism by which CD14 participates in the endocytosis of Pam_3CSK_4. CD14 is a GPI-linked protein with no cytoplasmic domain to facilitate the activation of endocytic signaling pathways. Thus, it is unclear how CD14 affects the endocytosis of TLR ligands. CD14 and TLR2 are both localized to lipid rafts upon ligand recognition (193, 208, 209), and the TLR2/6 ligand lymphotoxin alpha (LTA) is internalized through lipid raft-dependent mechanisms (208). It is therefore tempting to speculate that CD14 participates in the endocytosis of Pam_3CSK_4 by mediating the clustering of Pam_3CSK_4, TLR2/1, and integrin $\alpha_3\beta_1$ in lipid rafts. Once clustered, integrin $\alpha_3\beta_1$ may then signal for the endocytosis of Pam_3CSK_4 and TLR2/1. However, further work is necessary to examine this hypothesis.

Finally, Gerold et al. proposed integrin $\alpha_V\beta_3$ as a co-receptor for TLR2 (65) that regulates TLR2-mediated inflammatory responses. Using integrin α_V-targeting shRNA and blocking antibodies, we have now confirmed this finding (data not shown). However, the mechanism by which integrin $\alpha_V\beta_3$ cooperates with TLR2/1 remains unknown. It is tempting to speculate that integrin $\alpha_v\beta_3$ is responsible for attachment of Pam_3CSK_4 to the surface of the macrophages, since Gerold et al. hypothesized that integrin $\alpha_v\beta_3$ mediates attachment of bacterial lipopeptides to

cell surfaces (65). Further investigation is required to determine the exact mechanism by which integrin $\alpha_V\beta_3$ influences TLR2/1 signaling.

Future work is also necessary to understand the hypothesized interactions between integrin $\alpha_3\beta_1$, TLR2, CD14, integrin $\alpha_V\beta_3$. It is currently unknown which of these receptors interact to facilitate TLR2 signaling from the plasma membrane, and which of these receptors interact to facilitate signaling of endosomal TLR2/1. Furthermore, whether interactions at the plasma membrane or in the endocytic pathway form a multi-receptor complex, or whether these interactions are transient and sequential, remains unknown. There is some evidence to suggest that CD14 and TLR2/1 interact with Pam_3CSK_4 in a sequential fashion (139), but when and how integrin $\alpha_3\beta_1$ interacts with these receptors or with Pam_3CSK_4 remains unknown. Further investigation is therefore necessary to understand the nature of these interactions, and to better understand how these receptors cooperate in the cellular response to Pam_3CSK_4.

The importance of integrin $\alpha_3\beta_1$ in the recognition of bacterial lipopeptides and host defense in an *in vivo* model is unknown. Mice with integrin α_3 deficiency die early after birth and, to our knowledge, there are no cohorts of human subjects deficient in integrin $\alpha_3\beta_1$. Patients with leukocyte adhesion deficiency type III (LAD III) harbor a mutation in the KINDLIN3 gene which inhibits the activation of members of the β_1, β_2, and β_3 integrin families (137). These patients are highly

susceptible to multiple different infections. Whether the increased susceptibility to infection is caused by loss of integrin $\alpha_3\beta_1$ function specifically will require further research.

While the inflammatory response is necessary for the control of infection, this same response is thought to be primarily responsible for the clinical manifestations of Lyme disease. Thus, the host must employ mechanisms of immune regulation to control this inflammatory response, and protect itself from inflammation-mediated tissue damage. The particular mechanisms by which the mammalian host regulates this *B. burgdorferi*-induced inflammatory response are only beginning to be understood. In Chapter 4, we have demonstrated two novel findings. First, the anti-inflammatory peptide adrenomedullin is induced in response to *B. burgdorferi* both *in vivo* in the joints of infected patients, and *in vitro* in macrophage cell cultures. Second, adrenomedullin down-regulates the inflammatory response to *B. burgdorferi in vitro.* This second finding is significant for our understanding of *B. burgdorferi* pathogenesis, but also for our understanding of adrenomedullin biology. Prior to our studies, adrenomedullin has never been shown to regulate the inflammatory response induced by a multi-receptor stimulus.

Our current model for the induction of adrenomedullin and its role in modulating the *B. burgdorferi*-induced inflammatory response is shown in Figure 5.2. The recognition of *B. burgdorferi* by PRRs results in the induction of an inflammatory

response and the secretion of inflammatory cytokines, including TNF-α. TNF-α has been demonstrated by others to induce the expression of adrenomedullin (90, 124). Preproadrenomedullin mRNA is expressed and processed into the immature adrenomedullin peptide. This peptide is constitutively secreted from the cell, and activated through amidation in the extracellular space. This mature and active adrenomedullin binds its receptor, triggering unknown signaling cascades within the cell. These signaling events ultimately lead to the decreased expression of inflammatory cytokines such as IL-6, TNF-α, and IL-1β, and the increased expression of anti-inflammatory cytokines such as IL-10.

Figure 5.2

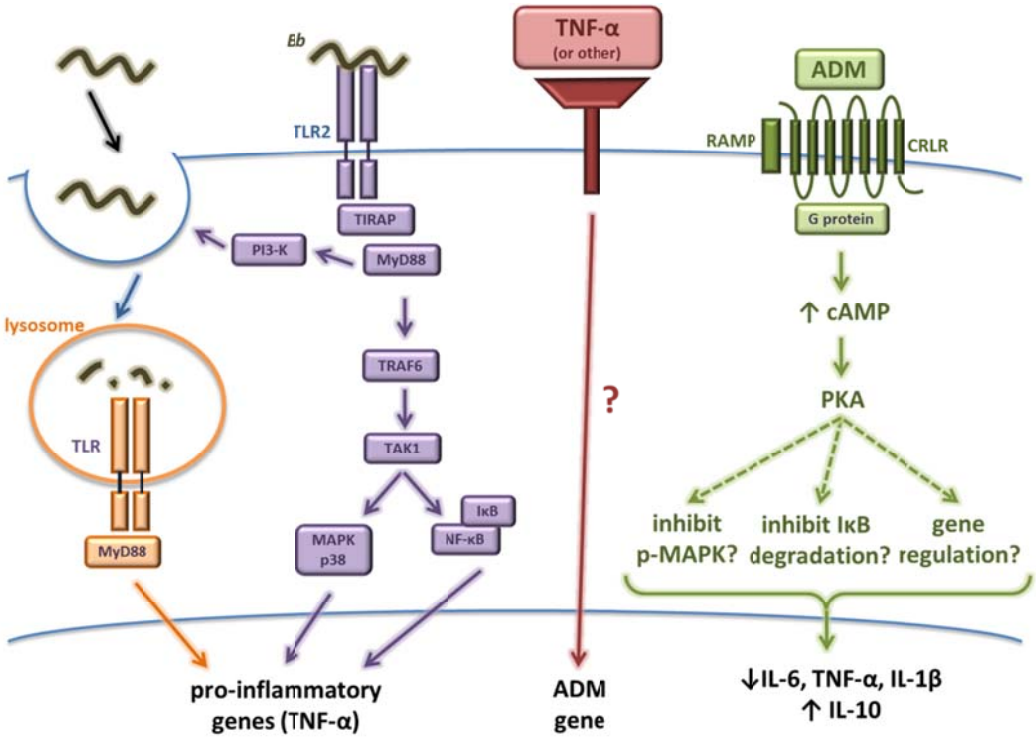


Figure 5.2 Model of adrenomedullin induction and regulation of *B. burgdorferi*-induced inflammatory responses

B. burgdorferi is recognized through the ligation of PRRs, such as TLRs, at the plasma membrane and endosomal compartments. These receptors activate signaling pathways that induce inflammatory cytokines, including TNF-α. TNF-α (or other cytokines) binds its receptor and, through unknown signaling events, induces the expression of adrenomedullin (ADM). Once secreted and activated, adrenomedullin interacts with its receptor complex, composed of calcitonin receptor-like receptor (CRLR) and receptor activity modifying protein (RAMP). The unknown signaling mechanisms activated by CRLR-RAMP result in the decreased expression of inflammatory cytokines such as IL-6, TNF-α, and IL-1β, and the increase of anti-inflammatory cytokines such as IL-10.

Whether adrenomedullin is among the anti-inflammatory molecules induced in response to *B. burgdorferi* has never been examined. Here, we show that levels of adrenomedullin are increased in Lyme arthritis compared with levels in the less-inflammatory osteoarthritis, suggesting that *B. burgdorferi* infection results in the increased production of adrenomedullin. In addition, levels of adrenomedullin in Lyme arthritis are significantly lower than those seen in rheumatoid arthritis. Interestingly, this indicates a correlation of adrenomedullin levels with the typical severity of inflammation seen with the different forms of arthritis. Clinically, levels of inflammation with Lyme arthritis (as measured by leukocyte infiltration) are typically intermediate between that seen in rheumatoid and osteoarthritis although there is considerable variability in the levels of inflammation seen in each condition. Our data are therefore consistent with previous reports that the production of adrenomedullin is up-regulated under inflammatory conditions (87, 90, 124, 210). The correlation of adrenomedullin production with severity of arthritis suggests that, at a minimum, adrenomedullin is insufficient to completely control the inflammatory response. Whether inflammation in rheumatoid arthritis or Lyme arthritis would be more severe in the absence of adrenomedullin or whether additional adrenomedullin would be useful in further dampening inflammation remains unknown.

Using *in vitro* models, we have also shown that the co-culture of *B. burgdorferi* with murine RAW264.7 macrophages induces the expression of adrenomedullin in a dose-dependent manner. Previous work done by our laboratory and others

has identified several major signaling pathways that are responsible for the regulation of the macrophage response to *B. burgdorferi* including MyD88, PI3-K, and p38 (7, 16, 21, 104, 188, 189). MyD88 and PI3-K are important for the phagocytosis of the spirochetes (188, 189), and this phagocytosis is in turn crucial for the induction of pro-inflammatory cytokines (49, 188). In addition, we and others have demonstrated a role for p38 MAP kinase in the inflammatory response to *B. burgdorferi* (7, 19). Signaling through p38 is associated with TLR-mediated responses, and is involved in the up-regulation of various pro-inflammatory molecules. We therefore hypothesized that inhibition of these molecules, and therefore known cellular responses to *B. burgdorferi*, may also inhibit subsequent adrenomedullin expression. Our data demonstrates that adrenomedullin expression in response to *B. burgdorferi* co-culture is inhibited in MyD88$^{-/-}$ macrophages, as well as by Ly294002 and SB203580, widely-accepted inhibitors of PI3-K and p38, respectively. Taken together, our data suggest that the established mechanisms for the induction of the inflammatory response are also necessary for the later activation of adrenomedullin. This co-regulation of pro- and anti-inflammatory processes may help to guarantee the control of the potentially deleterious inflammatory response. One caveat to these results is that neither of Ly294002 nor SB203580 is completely specific for their primary target kinases, complicating interpretation of this data (10, 21, 117). Unfortunately, at this time, completely specific inhibitors of PI3-K and p38 are not commercially available.

The induction of adrenomedullin by *B. burgdorferi* occurs later than the first wave of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 which are increased within hours in response to bacterial ligands. In the case of TNF-α, induction often begins to decrease by as early as 4-6 hours after exposure despite the ongoing presence of bacteria ((55) and data not shown). The late induction of adrenomedullin after incubation of cells with *B. burgdorferi*, as well as the fact that an inhibitor of new protein synthesis (cycloheximide) reduces the induction of adrenomedullin mRNA, suggest that *B. burgdorferi*-induced inflammatory molecules, rather than *B. burgdorferi* itself, stimulate the expression of adrenomedullin. Indeed, previous reports have shown TNF-α to be sufficient for the induction of adrenomedullin expression (90, 124). Later induction of an anti-inflammatory molecule such as adrenomedullin by the early pro-inflammatory molecules would allow for the early development of inflammation, which may be required for control of infection. Then, after the initial burst of inflammation, induction of adrenomedullin and other anti-inflammatory molecules at later time points may serve to dampen inflammation and the associated tissue damage, allowing for more targeted control of inflammatory processes.

Many studies have demonstrated the anti-inflammatory properties of adrenomedullin both *in vitro* (102, 112, 147, 218) and *in vivo* (32, 70-72, 160, 205, 223, 224). However, each of these experimental models involved the activation of cells through a single receptor (i.e., LPS stimulating the production of inflammatory cytokines through TLR4 (218)). Prior to our studies, the ability of

adrenomedullin to down-regulate an inflammatory response induced by a whole bacterium that activates cells through numerous receptors had never been explored. The addition of adrenomedullin to the media of RAW264.7 cells co-cultured with *B. burgdorferi* significantly decreased the production of inflammatory cytokines. These data suggest that the immunomodulatory activity of adrenomedullin is sufficient to down-regulate the expression of inflammatory mediators induced by whole *B. burgdorferi*.

The mechanisms by which adrenomedullin negatively regulates inflammatory responses have not been fully defined. Mature and active adrenomedullin transmits signals through a G protein-coupled CRLR-RAMP complex, but the signal transduction pathways downstream of this receptor are not well defined. One group has suggested that adrenomedullin-activated signaling pathways inhibit MAPK activity (226). Another group has proposed that adrenomedullin induces the up-regulation of peroxisome proliferator-activated receptor-γ (PPAR γ), which in turn inhibits IκB degradation and NF-κB translocation to the nucleus (147). However, even when a mechanism is proposed, the signaling pathways are not fully elucidated. Further work will be required to understand the precise signaling pathways that activate the transcription of adrenomedullin, as well as the signaling pathways activated downstream of the CRLR-RAMP receptor complex to affect the down-regulation of inflammatory processes.

The effect of adrenomedullin on inflammation *in vivo* remains unknown. The effect of exogenously-administered adrenomedullin in dampening inflammation in a mouse model of rheumatoid arthritis has been reported (70). This model used a single intraperitoneal injection of rat adrenomedullin into mice that were then given intra-articular collagen injections to stimulate arthritis. However, similar experiments in a murine model of Lyme arthritis would be complicated by several caveats. Unlike the collagen-induced model of rheumatoid arthritis, murine Lyme arthritis develops over a period of 3-4 weeks, and treatment with a single dose of adrenomedullin is unlikely to be sufficient to control the inflammatory response over such a long span of time. Furthermore, the availability of only species-incompatible adrenomedullin limits the potential for repeated administration due to the development of a reaction to the foreign protein. An alternative to administering exogenous adrenomedullin to *B. burgdorferi*-infected mice would be to determine the effects of the absence of adrenomedullin in a knockout mouse model. However, deletion of the adrenomedullin gene results in embryonic lethality (31). These studies await the generation of conditional knock-out mice which lack the expression of adrenomedullin in the joints and monocyte cells.

In summary, we have demonstrated that the induction of a complete inflammatory response to bacterial lipopeptides is mediated through integrin $\alpha_3\beta_1$. The mechanism by which integrin $\alpha_3\beta_1$ influences TLR2/1 signaling is by regulating the endocytosis of lipopeptides, and the subsequent recognition of

these ligands by TLR2/1 from within endosomal compartments. TLR2/1 signaling results in the activation of different downstream pathways from the plasma membrane and from endosomal compartments. We therefore propose a model in which integrin $\alpha_3\beta_1$ mediates the endocytosis of bacterial lipopeptides, thus facilitating the recognition of these ligands and subsequent initiation of signaling cascades by endosomal TLR2/1. This inflammatory response is regulated, in part, by the induction of adrenomedullin in response to *B. burgdorferi* both *in vivo* in the joints of infected patients, and *in vitro* in macrophage cell cultures. The induced adrenomedullin down-regulates the inflammatory response to *B. burgdorferi.* These studies strongly implicate a role for adrenomedullin in the modulation of inflammatory responses associated with infection with *B. burgdorferi*.

CHAPTER 6

MATERIALS AND METHODS

Cell cultures and reagents

The human monocyte cell line U937 (American Type Culture Collection) was maintained in RPMI (Mediatech) with 10% FBS (Atlanta Biologicals) and 1% penicillin-streptomycin (Mediatech). For all experiments, U937 monocytes were differentiated into macrophages at a concentration of $5x10^5$ cells per well in 24-well plates with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma) for 48 hours. All experiments were performed under serum-free conditions, unless otherwise stated.

RAW264.7 macrophage cell line was maintained at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech, Inc) containing 10% FBS and 1% penicillin-streptomycin. All experiments were performed under serum-free conditions, unless otherwise stated.

Bone marrow-derived macrophages from C57BL/6 mice and $MyD88^{-/-}$ mice backcrossed to C57BL/6 for 12 generations were recovered from mouse femurs and differentiated as described previously (188, 189). Briefly, bone marrow cells were flushed from femurs with DMEM and cultured on plastic Petri dishes for 5-7 days in DMEM media containing 20% FBS, 30% L929 cell-conditioned media, and 1% penicillin-streptomycin. Differentiated macrophages were then seeded in 24-well plates for experiments. The procedures used for these studies were reviewed and approved by Tufts University Institutional Animal Care and Use Committee.

Clonal isolates of infectious, low passage *B. burgdorferi* sensu stricto (strain N40, clone D10E9) were cultured in Barbour-Stoenner-Kelley (BSK II) medium at 37^{o}C as previously described (12).

The TLR2/1 triacylated lipid ligand, Pam_3CSK_4 (Invivogen), biotinylated Pam_3CSK_4 (Axxora), or Pam_3CSK_4-rhodamine (Invivogen) were resuspended in endotoxin-free water. Pam_3CSK_4 was used at a concentration of 100 ng/ml, and Pam_3CSK_4-biotin and Pam_3CSK_4-rhodamine were used at indicated concentrations.

Recombinant, mature, amidated rat adrenomedullin (American Peptide) was resuspended in endotoxin-free water and stored in small aliquots at -80°C. In cell culture experiments, adrenomedullin was added at a concentration of 100 nM, which was chosen based on prior studies (113, 124, 147, 218). This concentration of adrenomedullin does not affect the viability of RAW264.7 macrophages, as determined by trypan blue exclusion assay.

shRNA

Lentiviral plasmid vectors (pLKO.1) encoding non-targeting shRNA, integrin α_3-targeting shRNA, or TLR2-targeting shRNA (Sigma-Aldrich) were packaged into lentiviral particles following the manufacturer's instructions. Briefly, HEK293 cells were transfected with shRNA vectors and packaging vectors (Sigma-Aldrich) using FuGENE6 (Roche). Supernatants were harvested 48 and 72 hours post-

transfection and stored at -80°C. To reduce expression of target genes in U937 cells, the monocytes were incubated with lentiviral particles for 20 hours at 37°C. The media was then replaced with fresh RPMI with 10% FBS for 24 hours. Positively transduced cells were selected with 6 μg/ml puromycin (Sigma-Aldrich) for 24 hours. These cells were then differentiated as described above. After differentiation, the cells were harvested in TRIzol (Invitrogen), and mRNA was isolated following the manufacturer's instructions. From this mRNA, cDNA was synthesized and qRT-PCR analysis was performed to determine the relative expression of integrins and TLRs. Of the five different gene-specific shRNA constructs tested, the data presented in this dissertation were obtained with the construct which best reduced expression of the target mRNA with no impact on other homologous molecules. The sequences of the shRNA constructs are as follows: integrin α_3 5' - CCTCTATATTGGGTACACGAT -3', TLR2 5'-CCCATGTTACTAGTATTGAAA -3'. In each experiment performed, some cells were examined by qRT-PCR to confirm the reduction of the expression of target genes.

Inhibitors and Blocking Antibodies

Inhibition of endosomal acidification was achieved using inhibitors of V-ATPase, concanamycin A (100 ng/ml) and bafilomycin A1 (500 μM) or the ionophore monensin (1 μM) (Sigma). Clathrin-mediated endocytosis was inhibited with CPZ (5 μM) (Sigma). Concentrations were chosen based on prior studies (84, 140).

These inhibitors were added 30 minutes prior to stimulation. For CPZ experiments, the media was replaced at the time of stimulation to remove CPZ.

Ly294002 and SB203580 (EMD Biosciences/Calbiochem) were resuspended in DMSO following the manufacturer's instructions. Cycloheximide (Sigma-Aldrich) was resuspended in endotoxin-free water following the manufacturer's instructions. The concentrations of Ly294002 (10 μM) and SB203580 (3 μM) were previously published (19, 189) and had no visible cytotoxic effects on the RAW264.7 cell cultures as described (19, 189). Cycloheximide was added to cell cultures at 5 μM, and had no effect on cell viability as determined by trypan blue exclusion assay.

For blocking antibody experiments with mouse ascites fluid, U937 macrophages were incubated for 2 hours prior to stimulation with 50 μg/ml control mouse ascites fluid (NS-1 murine myeloma, Sigma-Aldrich) or anti-integrin $\alpha_3\beta_1$ monoclonal antibody (P1B5, Millipore).

ELISA

Supernatants were collected 6 hours post stimulation. Human TNF-α and IL-6, and murine TNF-α, IL-6, and IL-1β, were measured using the DuoSet enzyme linked immunoabsorbent assay (ELISA) kit (R&D systems) following the manufacturer's instructions.

Quantitative Real-Time PCR (qRT-PCR)

RNA was extracted from cells using TRIzol (Invitrogen) following the manufacturer's instructions. RNA was resuspended in water containing RNaseOut Recombinant RNase inhibitor (Invitrogen). RNA was treated with DNaseI using the Turbo DNA-free kit (Ambion). cDNA was synthesized using the ImPromII kit (Promega) following the manufacturer's instructions. Quantification of cDNA was performed by qRT-PCR (iCycler, BioRad) using the iQ SYBR Green Supermix (BioRad). Cycling parameters were 95^{o}C for 15 minutes, followed by 40 cycles of 95^{o}C for 30 seconds, 60^{o}C for 30 seconds, and 72^{o}C for 30 seconds.

The primers used to detect human gene expression are as follows: IL-6, TNF-α, and β-actin were published previously (17, 18) and are as follows: Human IL-6, F: 5'-ATGAACTCCTTCTCCACAAGCGC-3', R: 5'-TGCCGTCGAGGATGTACCGA-3'. Human TNF-α F: 5'-AGGCGCTCCCCAAGAAGACA-3', R: 5'-AGGCTTGTCACTCGGGGTTC-3'. Human β-actin, F: 5'-CCACACCTTCTACAATGAGCTGCG-3', R: 5'-CGGAGTCCATCACGATGCCA-3'. Human integrin α_3 primers, F: 5'-CCCGCTATTATCAGATCATGCC-3', R: 5'-CAGTAGTATTGGTCCCGAGTCT-3' were generated by Primer Bank, ID# 6006011a1. Human integrin α_v primers, F: 5'-AATGTGACTGGTCTTCTACCCG-3', R: 5'-ACCACTGATGGGACTTAAATTCC-3' were generated by Primer Bank, ID# 4504763a2. Human integrin α_5 primers, F: 5'-TTCTGGAGTATGCACCCTGC-3',

R: 5’-TGGTCCACCTAAAACCACACG-3’ were generated by Primer Bank, ID# 4504751a3. Human integrin α_6 primers, F: 5’-TCGGCACAGCAACCTTGAA-3’, R: 5’-TTGTGAGACTCCTTTTCCAATC-3’ were generated by Primer Bank, ID# 30046796a2. Human TLR2 primers, F: 5’-CCAGCACACGAATACACAGT-3’, R: 5’- CAAATGAAGTTATTGCCACC-3’. Human TLR4 primers, F: 5’-TACAAAATCCCCGACAACCTCC-3’, R: 5’-GCTGCCTAAATGCCTCAGGG-3’ were generated by Primer Bank, ID# 19924149a1. Human TLR7 primers, F: 5’-GGAACGGGTACCAAAATGGTGTTTCCAATGTGG-3’, R: 5’-TAATCTGGATCCGACCGTTTCCTTGAACACCTG-3’. Human TLR9 primers, F: 5’-GCGACCAGGCTCCCGAAGG-3’, R: 5’-GTGTCCTTTGCCCACCTGTCTC-3’. Human IFN-α1 primers, F: 5’-GCCTCGCCCTTTGCTTTACT-3’, R: 5’-CTGTGGGTCTCAGGGAGATCA-3’ were generated by Primer Bank, ID# 13128950a1 (194, 213). Primers used to measure IFN-β were published previously (230).

The primers used to detect murine gene expression are as follows: TNF-α, MCP-1, and CXCL-2 were published previously (16) and are as follows: Murine TNF-α, F: 5’-ATGAGCACAGAAAGCATGATC-3’, R: 5’-TACAGGCTTGTCACTCGAATT-3’. Murine MCP-1, F: 5’-GCTGTTCACAGTTGCCGGCT-3’, R: 5’-CATTAGCTTCAGATTTACGG-3’. Murine CXCL-2, F: 5’-GGGTTGACTTCAAGAACATCCAGA-3’, R: 5’—GGGCTTCAGGGTCAAGGCAAAC-3’. The sequences of the primers used to detect IL-6, IL-1β, β-actin, and adrenomedullin are as follows: Murine IL-6 F: 5’-

GACTTCACAGAGGATACCAC-3', R: 5'-TATCCAGTTTGGTAGCATCC-3'. Murine IL-1β F: 5'- TGGACCTCTGCCCTCTGGAT-3', R 5'-CATCGTGCACATAAGCCTCGTT-3'. Murine β-actin F: 5'-GTGCGTGACATCAAAGAGAAGC-3', R: 5'- GATGCCACAGGATTCCATACCC-3'. Murine adrenomedullin F: 5'-CACCCTGATGTTATTGGGTTCA-3', R: 5'-TTAGCGCCCACTTATTCCACT-3'.

Expression of target genes was referenced to expression of β-actin. Calculations of expression were normalized using the $\Delta\Delta C_t$ method, where the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta Ct}$, where C_t is the cycle number of the detection threshold.

Ligand Tethering Experiments

High sensitivity streptavidin-coated plates (Pierce, ThermoScientific) were coated with 5 μg/ml Pam_3CSK_4-biotin in PBS or control, and washed prior to the addition of U937 macrophages. In control wells, soluble Pam_3CSK_4-biotin was added after allowing U937s cells to settle. In experiments with blocked wells, wells were coated with 20 μg/ml biotin-HRP (Invitrogen) in PBS or control, and washed prior to the simultaneous addition of U937 macrophages simultaneously with 5 μg/ml Pam_3CSK_4-biotin.

Endocytosis Assay

For Pam_3CSK_4 endocytosis experiments, U937 monocytes stably transduced with integrin α_3-targeting shRNA or control shRNA were differentiated in wells containing glass coverslips. After differentiation, 5 ug/ml Pam_3CSK_4-biotin or Pam_3CSK_4-rhodamine were added. At the indicated time points, the cells were washed three times in cold PBS, fixed in 3.7% paraformaldehyde, and stained for immunofluorescent microscopy.

B. burgdorferi endocytosis experiments were performed according to a similar protocol. U937 monocytes stably transduced with integrin α_3-targeting shRNA or control shRNA were differentiated in wells containing glass coverslips. After the addition of *B. burgdorferi*, the plates were centrifuged at 300 x g at 4°C for 5 minutes. After 60 minutes at 37°C, the cells were washed three times in cold PBS, fixed in 3.7% paraformaldehyde, and stained for immunofluorescent microscopy.

Microscopy

Immunofluorescent microscopy was performed as previously described (188, 189) with the following modifications. For Pam_3CSK_4 endocytosis experiments, macrophages on coverslips were incubated with an anti-biotin FITC-conjugated polyclonal goat antibody (Novus Biologicals) at a 1:500 dilution to label extracellular Pam_3CSK_4-biotin. Macrophages were then washed three times for 5 minutes in PBS and permeabilized with -20°C methanol. Macrophages were then

incubated with anti-biotin Texas Red-conjugated polyclonal goat antibody (Novus Biologicals) at a 1:500 dilution to label both extracellular and intracellular Pam_3CSK_4-biotin. The coverslips were mounted in Vectashield mounting medium (Vector Laboratories).

For *B. burgdorferi* endocytosis experiments, macrophages on coverslips were incubated with an anti-*B. burgdorferi* polyclonal rabbit antibody (gift from Dr. Allen Steere) at a 1:10,000 dilution, then washed and incubated with a FITC-conjugated goat anti-rabbit IgG antibody (Molecular Probes) to stain extracellular bacteria. Macrophages were washed three times for 5 minutes in PBS and permeabilized with -20°C methanol. Macrophages were again incubated with anti-*B. burgdorferi* antibody, followed by a Texas Red-conjugated goat anti-rabbit IgG antibody (Molecular Probes) to stain both extracellular and intracellular bacteria. Coverslips were mounted in Vectashield mounting medium.

Coverslips were examined using a Zeiss Axiolan 2 microscope. Images were captured with a digital CCD camera (Hamamatsu). Images were merged using Volocity software (Improvision, Inc.). The percent association was determined by dividing the number of macrophages with Pam_3CSK_4-biotin or *B. burgdorferi* associated (either external or internal) by the total number of macrophages. The percent endocytosis was determined by dividing the number of macrophages that had internalized Pam_3CSK_4-biotin or *B. burgdorferi* by the number of macrophages associated with Pam_3CSK_4-biotin or *B. burgdorferi*.

For confocal microscopy studies, macrophages were permeabilized and incubated with an anti-TLR2 antibody (clone TLR2.1, Invivogen) at 1:50 dilution, then washed and incubated with an anti-mouse Alexa Fluor 488-conjugated secondary antibody to detect TLR2.

Confocal microscopy was performed at the Tufts Imaging Core Facility using the Leica TCS SP2 AOBS microscope using the Argon 488nm and HeNe 568nm red diode lasers. For simultaneous green and red channel imaging, the multitracking function was utilized and each laser was activated one at a time, ensuring no cross-talk occurred between the two fluorochromes. Z plane images of 0.7 μm were captured using the 63X oil objective and analyzed using the Leica Confocal Software (Leica).

Amnis Image Stream

HEK293 cells were incubated with 5 μg/ml Pam_3CSK_4-rhodamine in 24-well plates for 60 minutes under serum-free conditions. The cells were incubated on ice for 5 minutes, and washed with ice-cold PBS to remove the cells from the plates. The cells were pelleted at 300 x g for 10 minutes, and resuspended in 50 μl cold PBS. The cells were imaged with Image Stream (Amnis Corporation), and analyzed using IDEAS software 3.0 (Amnis Corporation) to determine the percent endocytosis. For each cell, the Image Stream captures an image of 10 μm depth through the center of the cell. The IDEAS software identifies the outline of the cell based on the brightfield image. From this outline, an “internal mask” is

determined and delineated from the “surface mask” of the cell. The fluorescence of the “surface mask” is excluded from analysis, and thus only the internal fluorescence of each cell was quantified.

Patients

Synovial fluid from patients with untreated Lyme arthritis, osteoarthritis, or rheumatoid arthritis was used for these studies. The conduct of this study was reviewed and approved by the Tufts Medical Center Investigational Review Board. Synovial fluid was obtained from 14 patients with untreated Lyme arthritis. These samples have been previously described (131, 132). All patients met the Centers for Disease Control clinical criteria for the diagnosis of Lyme disease. They were infected in the northeastern United States, and had monoarticular or oligoarticular arthritis affecting at least one knee accompanied by a positive immunoglobulin G western blot test for Lyme disease interpreted according to the Center for Disease Control and Prevention/ Association of State and Territorial Public Health Laboratory Directors (CDC/ASTPHLD) criteria (1). All 14 patients had a positive PCR test for *B. burgdorferi* DNA in synovial fluid, performed as described by Nocton et al. (157). The mean duration of symptoms at the time of sample collection was 2.2 years (range, 6 months to 6 years).

Synovial fluid from rheumatoid and osteoarthritis patients were collected from knees of patients diagnosed with rheumatoid or osteoarthritis by a board certified

rheumatologist and met clinical and/or laboratory criteria for their diagnosis. All specimens were stored at –80°C until use.

Radioimmunoassay

The concentration of adrenomedullin in synovial fluid was determined by radioimmunoassay (Bachem) following the manufacturer's instructions with the following modification: due to the small volume of Lyme arthritis synovial fluid available for use in this assay, the volumes of all samples and reagents were reduced by 5-fold. Briefly, undiluted synovial fluid was incubated with anti-adrenomedullin antibodies in 12x75 mm polypropylene tubes (Evergreen Scientific) overnight at 4˚C. Iodinated adrenomedullin tracer was added to each tube and incubated overnight at 4˚C. The adrenomedullin-antibody complexes were immunoprecipitated using secondary antibodies and normal rabbit serum. These pellets were read using a Micromedic gamma counter (Micromedic Systems).

Statistical analysis

For radioimmunoassay measurements, the median adrenomedullin concentration is represented by a horizontal line. Statistical significance was determined using Mann-Whitney U analysis.

For qRT-PCR and ELISA assays, the data are shown in two ways. First, mean cytokine expression or secretion from one representative experiment is reported,

with error bars representing the S.D. of replicates. Second, relative cytokine expression or secretion is reported (normalized to control samples), with error bars representing the S.E.M. of three or four independent experiments. For these data, mean expression or secretion in the control samples is arbitrarily set to 1.0 (for qRT-PCR) or 100% (for ELISA) in each experiment, and mean expression or secretion in the experimental samples is expressed as a fraction (qRT-PCR) or percentage (ELISA) of the control samples. Statistical significance was determined using Mann-Whitney U analysis.

For endocytosis experiments, the data are shown in two ways. First, mean percent association or mean percent endocytosis from one representative experiment is reported. Second, relative percent association or relative percent endocytosis is reported (normalized to control samples), with error bars representing the S.E.M. of three independent experiments. For these data, the mean percent association or mean percent endocytosis in the control samples is arbitrarily set to 100% in each experiment, and the mean percent association or mean percent endocytosis in the experimental samples is expressed as a percentage of the control samples. Statistical significance was determined using Mann-Whitney U analysis.

For Amnis Image Stream experiments, mean percent endocytosis, with error bars representing the S.E.M. for three independent experiments. Statistical significance was determined using Mann-Whitney U analysis.

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