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Influence of tick transmission on the host response to Rickettsial infection

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INFLUENCE OF TICK TRANSMISSION ON THE HOST RESPONSE TO RICKETTSIAL INFECTION

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Abstract

Several members of the spotted fever group (SFG) of *Rickettsia* are transmitted to the host through infected ixodid (hard) ticks, which can serve as both vectors and reservoirs. Multiple studies have demonstrated that ticks secrete proteins into the bite site of the host that suppress innate and adaptive immune responses. While this suppression of immune responses is beneficial to the tick, it may also be beneficial to the transmitted *Rickettsia*. We hypothesize that *Rickettsia* utilize the tick's ability to alter the host immune response at the tick feeding site to successfully establish infection. In the current study, we analyzed how the tick transmission influenced the response to *Rickettsia* infection by comparing the innate immune response following intradermal versus tick-inoculation of *R. amblyommii* in the skin. In particular, we analyzed mRNA expression of Toll-like receptor (TLR) and TLR adaptor/effector genes as well as proinflammatory cytokines and chemokines. Data was analyzed using One-way ANOVA and Newman-Keuls post tests. The results of this study are the first to delineate the immunomodulatory milieu associated with rickettsial infection during the natural route of tick exposure.

Chapter 1: Introduction

     Rickettsia is a gram-negative bacterium that causes a variety of diseases, including Rocky Mountain spotted fever. The primary vectors for rickettsial transmission are ticks, which act as reservoirs and hosts. Infected ticks transmit *Rickettsia* to humans through their saliva during blood meal acquisition. Tick saliva present at the bite site contains multiple proteins that can influence innate and adaptive immune responses in the skin. However, the impact of tick transmission on the innate and adaptive immune response to *Rickettsia* infection is unknown. The primary cell type that recognizes bacterial infection in skin is the Langerhans cell (LC) a type of dendritic cell present in the epidermis of the skin. Immature LCs capture antigen, process antigen and then migrate to the draining lymph node to present antigen to T cells. As such, LCs act as a bridge between the innate and adaptive immune system. LCs detect bacterial infection by recognition of Pathogen-associated molecular patterns (PAMPs), repeated structures expressed by microbes and not normally found in vertebrates. These PAMPs are recognized by Pattern-recognition receptors (PRRs), expressed on LCs which include membrane-bound Toll-like receptors (TLRs) as well as cytoplasmic RNA-helicase and NOD proteins. Though bacterial recognition by LCs is considered the first line of immune reaction in skin, in-vitro studies have shown that some bacteria can suppress the innate immune response to avoid inflammatory reaction and early elimination by the host during bacterial infection. An analogous situation occurs during tick feeding, where ticks can feed for extended period of time on the vertebrate host without causing any significant immune response. This immune-suppression by ticks may influence *Rickettsia* transmission during tick blood meal feeding. In the present study, we analyzed the innate immune response during tick transmission of *Rickettsia* versus intradermal

(ID) inoculation of *Rickettsia*. Mice were either infested with uninfected ticks, infected ticks or injected ID with *Rickettsia*. Tissues were collected after five to seven days post infection and mRNA expression of a number of innate and adaptive immune response genes were measured using Real-time PCR. The hypothesis is that *Rickettsia* utilize the tick's ability to alter the host immune response at the tick feeding site to successfully establish infection. The objective of this study was to determine how tick transmission of *Rickettsia* altered the innate immune response compared to direct *Rickettsia* inoculation.

Chapter 2: Literature Review

2.1: *Rickettsia* and Rickettsioses

Rickettsia is a genus of non-motile, rod shaped, gram negative, obligate, intracellular bacteria of eukaryotic cells that replicate mainly inside the cytoplasm of endothelial cells. Rickettsiae belong to the order Rickettsiales and family Rickettcieae (Raoult and Roux, 1997). There are two antigenically defined groups of *Rickettsia*, Spotted fever group and Typhus group associated with human infection. In nature, rickettsioses are zoonoses and cause human diseases in United States and around the world (Baron, 1996), they are entirely arthropod-borne, with ticks, mites, lice and fleas being the main vectors. *Rickettsia* can cause a variety of diseases including Rocky Mountain spotted fever (RMSF) caused by *R. rickettsii*, rickettsial pox caused by *R. akari*, Oriental spotted fever caused by *R. japonica*, murine typhus caused by *R. typhi* and epidemic typhus caused by *R. prowazekii*. RMSF can cause certain neurological disorders, whereas other rickettsial diseases can result in pulmonary and renal failure, myocarditis and gangrene of the fingers, ear lobes, toes and external genitalia. Fatality can occur due to RMSF if the disease is not diagnosed at an early stage and if the patient is not treated promptly (Parola et al., 2005).

Ticks are the major vectors of *Rickettsia* and also serve as reservoirs. Ticks can transmit the bacteria during blood meal acquisition or through feces (Raoult and Roux, 1997). With the exceptions of epidemic typhus, humans are not mandatory hosts for maintaining the *Rickettsia* life cycle (Azad and Beard, 1998). Transmission from one tick to another can happen transovarially (from infected mother to its offspring) or transstadially (maintenance of infection throughout different stages of the tick life cycle) (Fig. 1). *Rickettsia sp*. remains in a dormant

stage in tick host and are activated during blood meal feeding. While feeding, the bacteria are transmitted to the vertebrate host through tick saliva and infect the endothelium lining the small blood vessel (Murray et al., 2005)
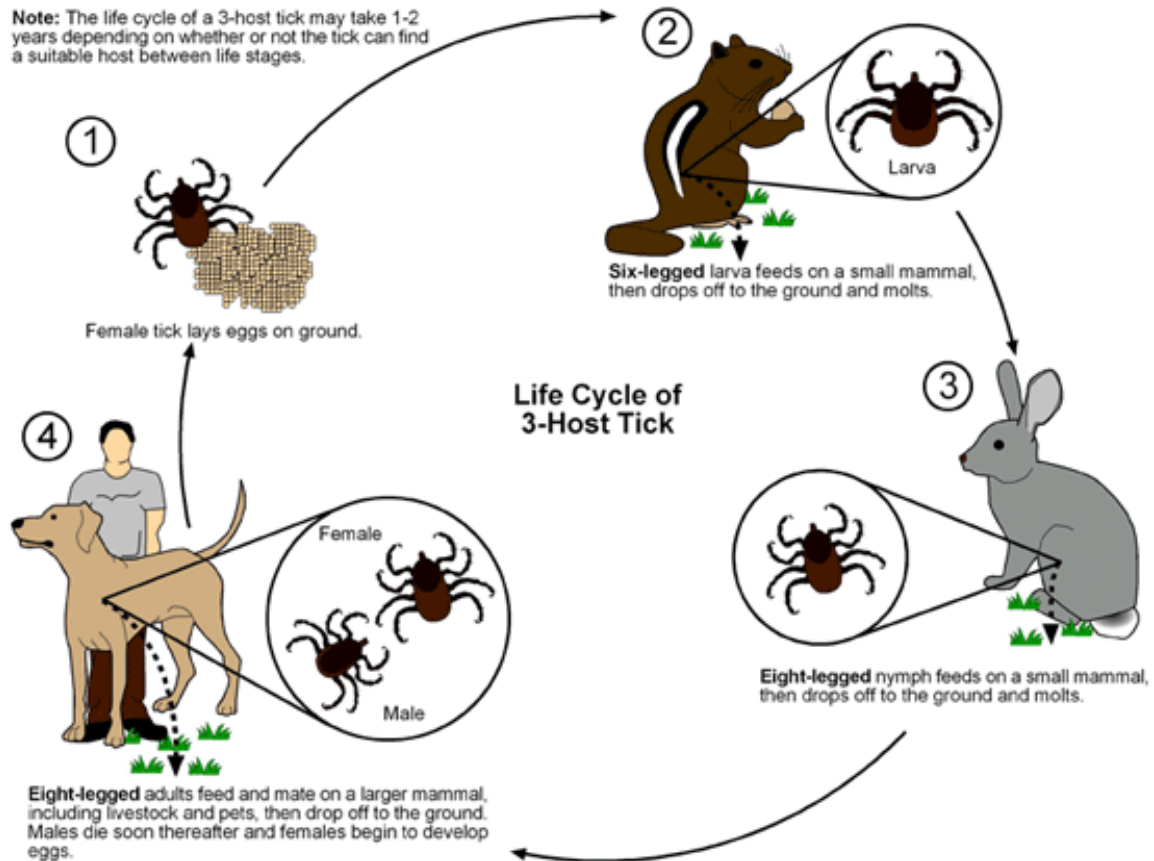
Fig. 1 Life cycle of Ixodid ticks. Taken from
http://www.entm.purdue.edu/publichealth/print/insects/tick.html

2.2: Rickettsial Pathogenesis and Host Defense Mechanism against Rickettsial Infection

Vertebrate hosts are infected with *Rickettsia sp.* entirely through tick bite. The clinical pathogenesis caused by *Rickettsia sp.* is purely from the disseminated infection and damage to the endothelial cells, which are the primary cell type infected. *Rickettsia sp.* replicates inside

endothelial cells and induces oxidative stress (Valbuena et al., 2002; Walker and Ismail, 2008). Rickettsial inoculation in skin through tick saliva can take two possible pathways to infection, and the severity of pathogenesis depends on which pathway is followed. Specifically, *Rickettsia* can either migrate directly to the capillaries and to lymphatic circulation or can migrate to the subcutaneous area of the skin. The former pathway leads to rapid dissemination of *Rickettsia*. The latter pathway activates the innate immune response via recognition of *Rickettsia* by Langerhans cell (LCs). Rickettsial infection also activates the endothelial cells which in turn induce the secretion of pro-inflammatory cytokines, chemokines, and adhesion molecules, suggesting that endothelial cells are associated with activation of the innate immune response (Valbuena et al., 2002). The primary cells acting against the rickettsial infection are natural killer cells, which produce interferon-gamma (IFN-γ). Tumor-necrosis factor alpha (TNF-α) and IFN-γ are the primary cytokines responsible for bacterial clearance and protecting the host against infection (Valbuena et al., 2002; Walker and Ismail, 2008).

The initiation of innate immune response to *Rickettsia* infection in the skin has not been extensively studied. There are many innate immune response molecules that are important during bacterial infection, and for recognition and response of host cells to infection. This includes several pattern recognition receptors (For example, TLRs, peptidoglycan receptor proteins or PGLYRPs, triggering receptor present on myeloid cells or TREM-1) and response molecules like Pro-platelet basic protein (Ppbp), heme-oxygenase-1(Hmox-1), interleukin-1beta (IL-1β), Caspase-1 (Casp-1), and Interleukin-6 (IL-6), which induce an array of immune response reactions protecting against bacterial infection. The main objective of the research project was to study the influence of tick transmission on the host response to rickettsial infection.

2.3: Role of Langerhans Cells in Immunity: Bridge between Innate and Adaptive Immunity:
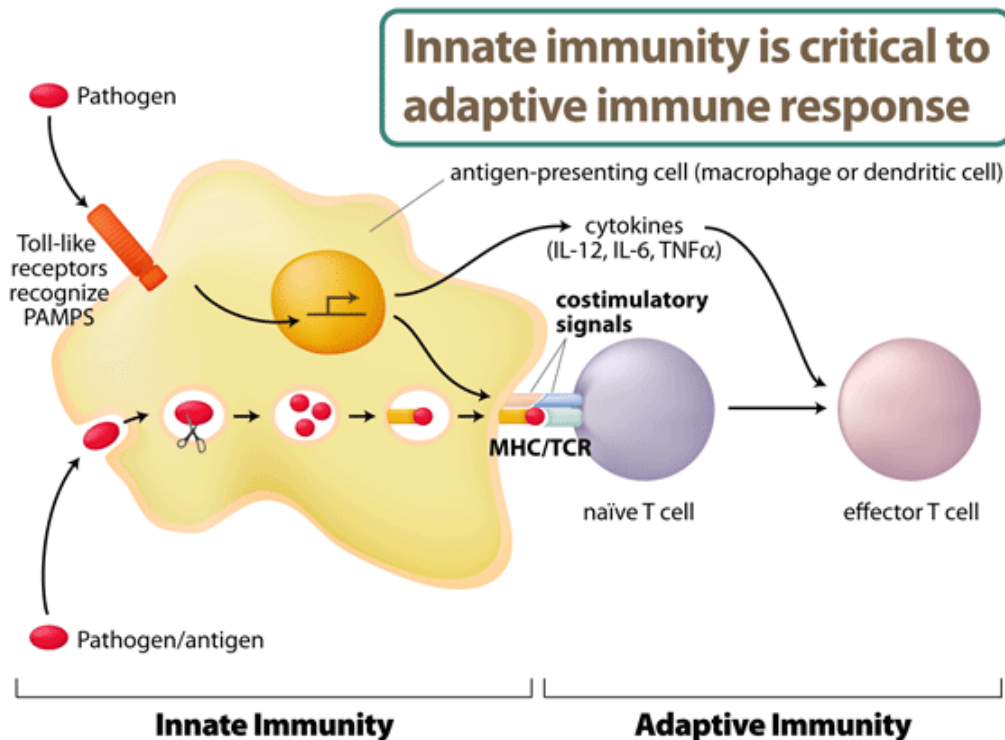
Fig. 2 Link between Innate and Adaptive Immunity.
Taken from http://research.dfci.harvard.edu/innate/innate.html

As a primary defense organ, skin represents a physical barrier between the environment and the body. The components in the skin that contribute to the immune response are collectively termed as Skin Associated Lymphoid Tissue (SALT) (Schwarz, 2003). Within the epidermal layer of skin, reside LCs, a subtype of dendritic cells (DC). LCs are characterized by the presence of Birbeck granules in their cytoplasm and presence of CD1a molecules. Langerin (CD207), which is a trans-membrane type-II C-type lectin, acts as a marker for LCs and DCs. Langerin can translocate lipids from bacterial cell wall to the Birbeck granules inside the cell (Valladeau et al., 2000; Koch et al., 2006). DCs detect components of different pathogens

through specialized cell surface receptors, PRRs or by macropinocytosis (Nakagawa and Bos, 2001), process the bacterial components and then migrate to T cell zones in the draining lymph node to activate the naïve T cells. The activation of T cells occurs through antigen presentation by major histocompatibility complex I and II on T cells (MHCI and MHCII) on DCs to T cell receptors on T cells as well as stimulation of CD80 and CD86 expressed on APCs (Fig. 2) (Moll, 2004).

Despite their role in pathogen recognition, LCs appear to have only poor recognition of invading bacteria in the skin. In vitro studies showed that LCs responded weakly to bacterial peptidoglycan (PGN) and do not respond to lipopolysaccharide (LPS) (Takeuchi et al., 2003). When LCs were challenged with LPS, and lipoteichoic acid were unable to up-regulate the maturation markers like CD86 and CD83 (van der Aar et al., 2007). In addition to poor responsiveness to bacterial product, some bacteria can suppress the maturation of DCs. For example, *Mycobacterium leprae* can suppress DC maturation/activation *in vitro* (Murray et al., 2007).

2.4: Pattern Recognition Receptors (PRRs) Involved in Pathogen Recognition and Inflammation

A plethora of immune responses are generated in response to microbial infection, all intended to eliminate the invading microbe (Figure. 3). Innate immunity to bacterial infection mainly relies on the recognition of PAMPs by PRRs (Kumagai et al., 2008). These PRRs can be classified into four groups: (i) TLRs, for example, TLR2, TLR4, TLR9 (ii) C-type lectin receptors (CLRs) for example, TREM-1, C-type lectin domain family 7, member A or CLEC7A (iii) NLRs (NOD [nucleotide binding and oligomerization domain]-like receptors and (iv) RLRs

(RIG-I [retinoic acid-inducible gene-1]-like receptors) (Palsson-McDermott and O'Neill, 2007); (Kumagai et al., 2008). Recognition of PAMPs by PRRs can activate several downstream signaling cascades and ultimately alter gene expression (Palsson-McDermott and O'Neill, 2007) to induce production of antimicrobials (Fig. 3).
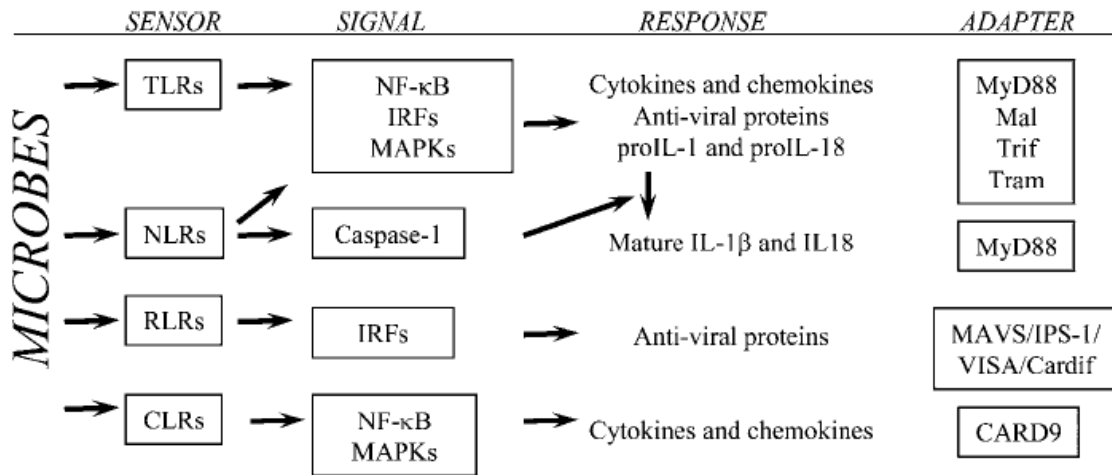
Fig. 3 Different PRRs, Their Signaling Molecules and Adaptor Molecules. Taken from Palsson-McDermott and O'Neill, 2007

2.4.1: Toll-like Receptors and Their Role in Innate Immunity

Toll-like receptors are one of the major PRR families of the innate immune system. Toll protein was first discovered in the fruit fly, *Drosophila* (Krutzik et al., 2001). Initial studies demonstrated a role for Toll in dorsal-ventral polarity in *Drosophila*, however later studies indicated that Toll contributes to anti-fungal immunity. Further studies identified 13 murine and 10 human "toll-like" receptors (Kumagai et al., 2008) and each TLR recognizes a specific bacterial or viral component. Structurally, TLRs are Type I trans-membrane proteins with the cytoplasmic portion very similar to IL-1 receptor, called the Toll-IL-IR or TIR domain. The

extracellular portion contains leucin-rich repeats (LRR) and differs from the IL-1R (Akira, 2001).

The LRR portion on TLRs recognizes PAMPs while the cytoplasmic portion is responsible for transducing the signal and activating transcription factor NF-κB, which ultimately leads to the induction of an array of pro-inflammatory genes (Shimazu et al., 1999). The LRR of different TLRs recognize different PAMPs. For example, TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria, TLR9 recognizes unmethylated DNA containing repeated CG motifs, and TLR2 recognizes bacterial peptidoglycan (Pasare and Medzhitov, 2004; Lebre et al., 2007; van der Aar et al., 2007).
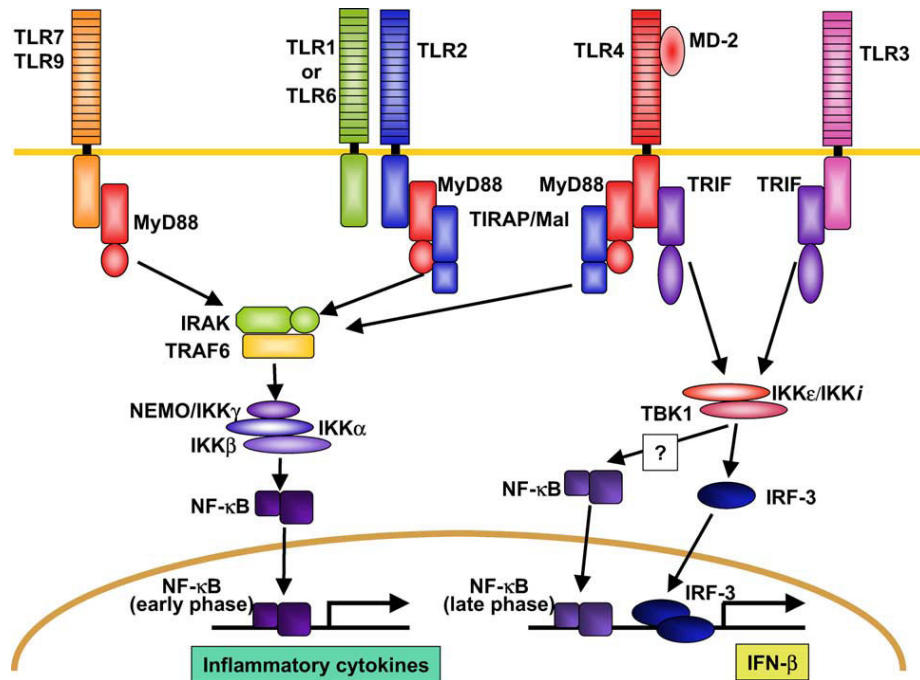
Fig. 4 Toll-like Receptors and Their Signaling Pathway. Taken from Takeda and Akira, 2004.

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Once TLRs recognize microbial components, they induce signals to activate other intracellular proteins including transcription factor NF-κB (Fig. 4). This signaling induces the up-regulation of molecules necessary for the maturation of the DCs (Pasare and Medzhitov, 2004). Microbial recognition by TLRs also induces DCs to produce co-stimulatory molecules like CD40 and cytokines and chemokines, for example, LPS or CpG DNA recognition and subsequent signaling can induce DCs to secrete IL-12.

TLR Signaling Pathway:

TLRs recognize different pathogens through their LRR domain, signal through the TIR domain and recruit downstream regulatory adaptor molecules to initiate signaling (Fig. 5). TLR initiate signaling through either the MyD88-dependent or MyD88-independent pathways (Muzio and Mantovani, 2001).
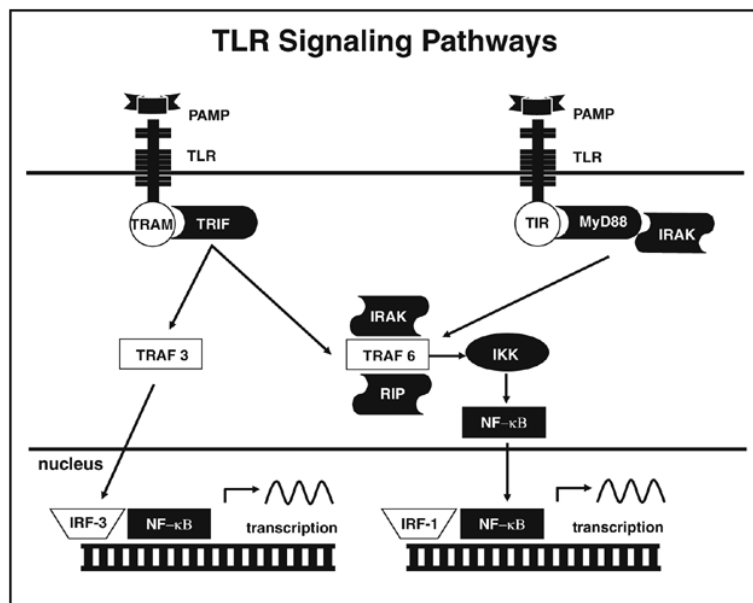
Fig. 5 Toll-like Receptor Signaling; Taken from Roses et al, 2008
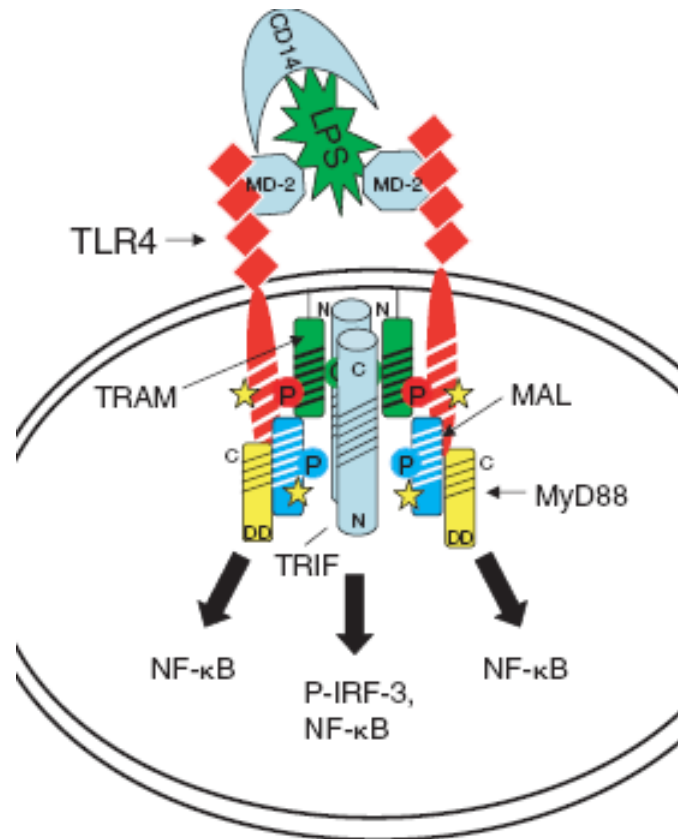
Fig. 6 Recognition of LPS via TLR4 and Activation of Both MyD88-dependent and Independent Pathway. Taken from Vogel et al, 2003.

TLR4 utilizes another pathway which is MyD88-independent and occurs through another adaptor molecule called TIR-containing adaptor protein (TIRAP) (Horng et al., 2001). TL4 can utilize both MYD88-dependent and independent pathway (Fig. 6) and the induction of MYD88-independent pathway by TLR4 leads to the secretion of IFN-β (Bagchi et al., 2007). A third adaptor molecule is also involved in the toll signaling called TIR domain-containing adaptor inducing IFN- β or TRIF. TLR3 can only utilize MYD88-independent pathway via TRIF and leads to the secretion of IFN-β, TNF- and IL-6 (Yamamoto et al., 2003; Bagchi et al., 2007).

Recognition of Bacterial Lipopolysaccharide by TLR4:

Gram-negative bacteria contain LPS as an outer membrane component and can cause endotoxic shock. The main component responsible for the biological activity of LPS is lipid A (Akira, 2001). Recognition of LPS in mainly mediated by TLR4, LPS- binding protein, CD14, and MD2 (Schnare et al., 2000; Miyake, 2007) and initiates signaling at the cell membrane (Ahmad-Nejad et al., 2002). Recognition of LPS via TLR4 complex induces oligomerization of TLR4, leading to further activation of downstream regulatory molecules.

LPS bound to the bacterial cell wall has poor immunogenicity. Instead, LPS released from the bacterial membrane binds lipid-binding protein (LBP), an acute phase protein, and acts like a shuttle protein (Miyake, 2007) and helps bring the LPS to macrophages. LBP can be either soluble or plasma membrane-bound (Fig. 7) (Miyake, 2007). LBP forms a complex with another host protein called CD14 (Dauphinee and Karsan, 2006). Certain types of bacterial LPS, *i.e* the smooth-form of LPS from the bacteria *Enterobactriaceae,* accessory molecule CD14 is required (Freudenberg et al, 2008) to transfer the LPS from LBP to TLR4. Other type of LPS do not require CD14 for delivery of LPS to TLR/MD2 complex (Dauphinee and Karsan, 2006).

TLR4 alone is not enough to initiate recognition to bacterial LPS; it requires another indispensible co-stimulatory molecule called Myeloid Differentiating factor 2 (MD2). MD2 is attached physically to the extracellular domain of TLR4 on the cell surface (Akira, 2001) together with TLR4, recognizes a variety of Gram-negative bacteria. Without CD14 or LBP, binding of LPS to the TLR4/MD2 complex is poor. MD2 regulates both LPS binding to TLR4 and TLR4 clustering (Miyake, 2007). TLR4 activation may lead to activation of both MyD88

dependent or MyD88-independent pathway and subsequently activation of NF-κB (Dauphinee

and Karsan, 2006).
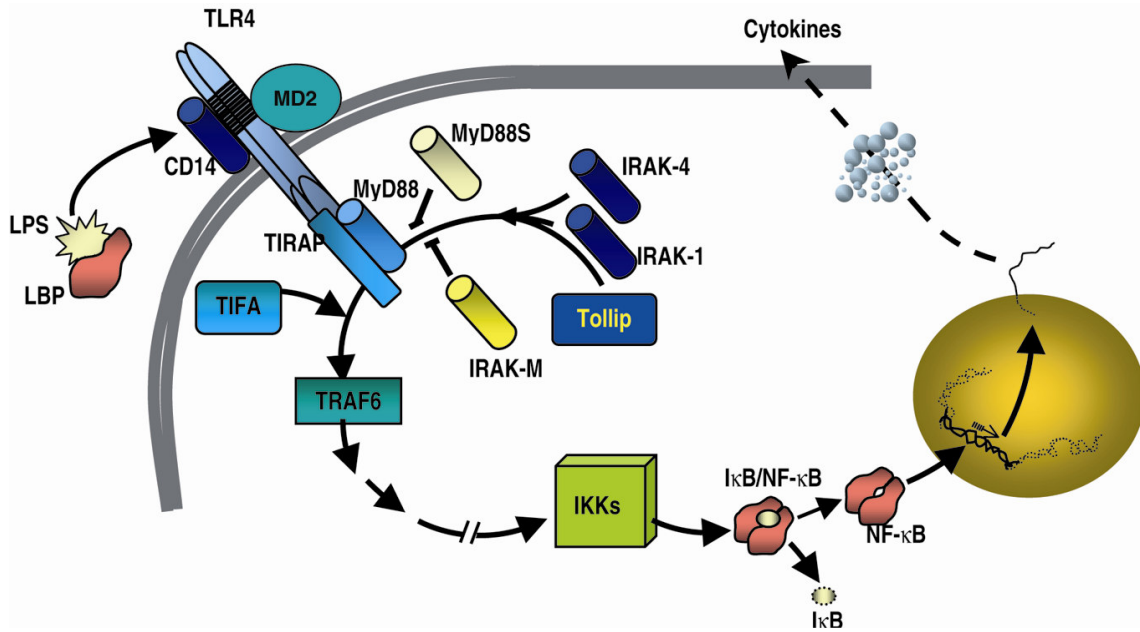
**Fig. 7 LPS Recognition through TLR4 and the MYD88-dependent Pathway of TLR
Signaling. Taken from Villar et al, 2004**

Recognition of LPS and PGN by TLR2:

TLR2 is present on the cell surface of many immune cells, like monocytes, macrophages,

and dendritic cells (Royet and Dziarski, 2007). TLR2 can recognize various bacterial cell wall

components, prominent among which are lipopeptides (LP), lipoteichoic acid, PGN and LPS.

TLR2 is unique among the TLRs in the sense that it signals as a heterodimer in association with

either TLR1 or TLR6 (Buwitt-Beckmann et al., 2006). Unlike TLR4, which recognizes only the

LPS monomer, TLR2 can recognize LPS when LPS is present only in large amounts. Like

TLR4, TLR2 also needs CD14 to initiate signaling after recognition of the PAMPs (Wetzler,

2003).

Recognition of Bacterial CpG DNA by TLR9:

Bacterial DNA has strong immunostimulatory effect and is able to stimulate proinflammatory cytokines. Bacterial DNA and vertebrate DNA differ highly in their CpG DNA content (CG island is a short stretch of DNA where "p" simply indicates that "C" and "G" are connected by a phosphodiester bond). In vertebrates, CpG motifs are flanked by two purines on the 5' and two pyrimidines on the 3' end. Another important difference is that vertebrate DNA is mostly methylated, whereas bacterial CpG DNA is unmethylated. The unmethylated CpG DNA motifs in the bacterial DNA act as PAMPs and are recognized by TLR9. The immunostimulatory activity of CpG DNA can activate the antigen presenting cells like dendritic cells, B cells and macrophages via MYD88-dependent signaling pathway (Schnare et al., 2000; Krieg, 2002). Unlike TLR2 and TLR4, TLR9 signaling is not dependent on CD14. Another difference between TLR2 and 4, and TLR9 is that TLR9 initiates signaling in the endosomal compartments only after endocytosis of CpG DNA (Ahmad-Nejad et al., 2002).

Negative Regulation of TLR Mediated Signaling by TOLLIP:

While TLRs are essential in controlling pathogen infection, the immune response also maintains a negative control to avoid any unnecessary reaction. TOLLIP is a small regulatory adaptor molecule in the TOLL pathway thought to regulate the TLR pathway by binding or interacting with the TIR domain of TLR2 and TLR4 (Wetzler, 2003; Didierlaurent et al., 2006). Tollip can also bind to IRAK1 and suppress the kinase activity. TOLLIP is constitutively expressed in unstimulated immune cells (Didierlaurent et al., 2006).

2.4.2: Peptidoglycan Recognition by Peptidoglycan Receptor Proteins (PGLYRPs)

Peptidoglycan is a key component of almost all bacteria, but not eukaryotic cells. Thus, peptidoglycan serves as a good target for pattern recognition (Dziarski and Gupta, 2006). The recognition of PGN is mediated by a receptor molecule called peptidoglycan receptor protein or PGRP (Liu et al., 2001), which is a PAMP recognition molecule and therefore belongs to the pattern recognition receptor family. PGRPs were first discovered in insects and later found in mammals. Initially the four forms of Peptidoglycan receptor protein were termed PGRP-S for 'short', PGRP-L for 'long', PGRP-1α and PGRP-1β for 'intermediate'. However, the names were recently changed to Peptidoglycan Recognition Protein 1, 2, 3 and 4 (PGLYRP1, PGLYRP2, PGLYRP3 and PGLYRP4) respectively for mouse and humans (Dziarski and Gupta, 2006; Lu et al., 2006). PGLYRPs kill bacteria by preventing cell wall peptidoglycan synthesis and by binding to the peptidoglycan precursor molecules (Dziarski and Gupta, 2006). PGLYRP1, PGLYRP3 and PGLYRP4 are constitutively expressed in areas that are always in contact with the environment including skin, oral epithelial cells, mucous membrane, and eyes, where they act as effective bactericides. PGLYRP1 is a soluble protein, present in bone marrow and almost exclusively expressed in the granules of polymorphonuclear leukocytes. PGLYRP2 is expressed in intestinal follicle-associated epithelial cells and is also constitutively expressed in liver where it is secreted from the liver in to the blood stream. It cleaves the stem peptide from the glycan chain of peptidoglycan (Lu et al., 2006). The expression of PGLYRP3 and PGLYRP4 in skin and oral epithelial cells increases in response to bacterial infection. The activation of PGLYRP3 and PGLYRP4 are thought to be mediated via TLR2, TLR4, Nod1, and Nod2 (Fig. 8) (Dziarski and Gupta, 2006).
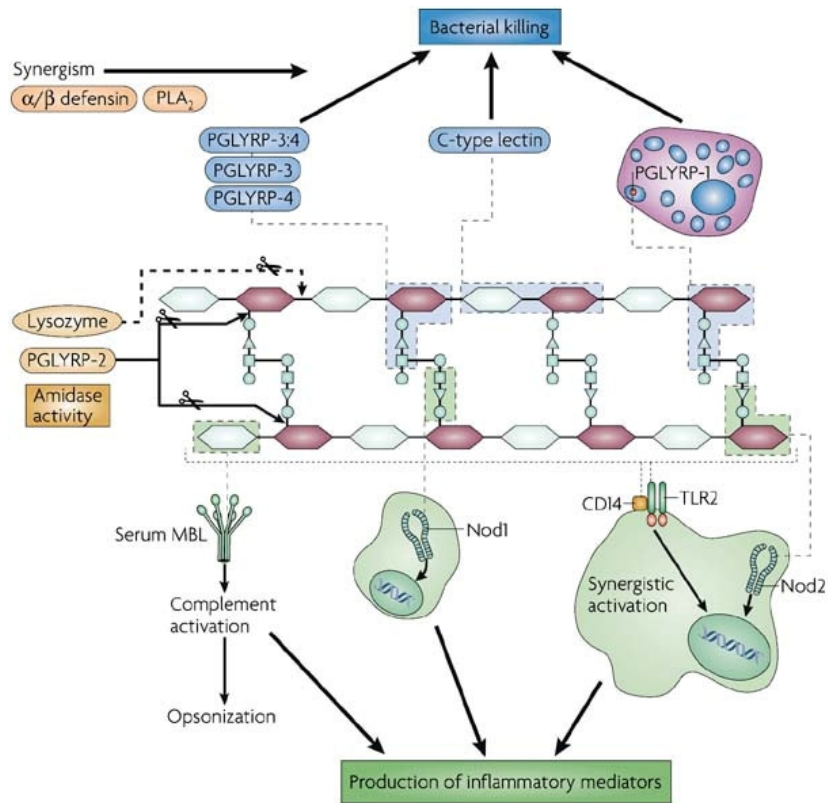
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Fig. 8 Recognition of Peptidoglycan. Taken from Royet & Dziarski, 2007

2.4.3: Triggering Receptor Present on Myeloid cells (TREM-1) as a PRR in Bacterial Infection

TREM-1, is a C-type lectin protein, is a member of the immunoglobulin super-family and is exclusively expressed on blood neutrophils and monocytes. Once bound to its ligands, *i.e* LPS or lipoteichoic acid, the intra-cellular domain of TREM-1 associates with the signal transduction molecule DAP-12, which is a TYRO protein tyrosine kinase binding protein. Monocytes and neutrophils up-regulate TREM-1 upon stimulation with LPS, heat-killed Gram-negative bacteria, Gram-positive bacteria, or fungi (Bouchon et al., 2000). LPS stimulation leads to TREM-1 mediated degranulation of neutrophils and induces secretion of several inflammatory chemokines

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and cytokines, including IL8, TNF-α and MCP-1, as well as up-regulate expression of adhesion molecules. TREM-1 is activated on cells stimulated via TLR ligands and TREM-1 silencing leads to the attenuation of the adaptor proteins MyD88, CD14 and IL-1β (Ornatowska et al., 2007), suggesting a role for TREM-1 in regulating TLR signaling.

2.4.4: Clec7a, Another PRR of Innate Immune System Recognizes Bacterial Component

Clec7a is a C-type lectin PRR expressed by several antigen presenting cells, including monocytes, macrophages and dendritic cells. Clec7a acts as an antigen binding molecule for APCs. Clec7a acts a receptor for carbohydrates in the bacterial cell wall in a calcium dependent manner, where the binding of carbohydrate is mediated by a conserved Ca^{2+} (Willcocks et al., 2006).

2.5: Recognition of Bacterial Components by PRRs Leads to Inflammatory Response Which Is Mediated by a Variety of Soluble Factors

2.5.1: TLR Activation Leads to the Activation of Caspase-1 and Subsequent Production of IL-1β

Activation of TLR2 and TLR4 leads to the activation of NF-κB and also activates a pro-apoptotic signal leading to cell death (Ruckdeschel et al, 2004). MyD88 contains a death domain (DD) and associates with Fas-associated death domain protein (FADD) to signal through NF-κB and activate the downstream apoptotic pathway. FADD can bind cytoplasmic cysteine protease Caspase-8 and initiate apoptosis. Caspase-1, a cysteine protease, also called IL-1β- converting enzyme (ICE) is constitutively synthesized as a 45-kDa pro-enzyme called pro-caspase-1. Pro-caspase-1 undergoes proteolytic cleavage to produce the mature enzyme, which takes place upon assembly of the "inflammosome" (Johansen et al., 2007), the caspase-activating complex (Martinon et al., 2002). Caspase-1 plays an important role in apoptosis and as well as in

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inflammation. TLR2 and TLR4 also activate Caspase-1 and helps in cell death by apoptosis and by releasing IL-1β from dying cells (Aliprantis et al., 2000). In monocytic and endothelial cells, LPS binds to LBP, transfer to the TLR4 via soluble CD14 molecule and induce the production of caspase-1, which in turn augments the synthesis of IL-1β gene and release of mature IL-1β (Schumann et al., 1998).
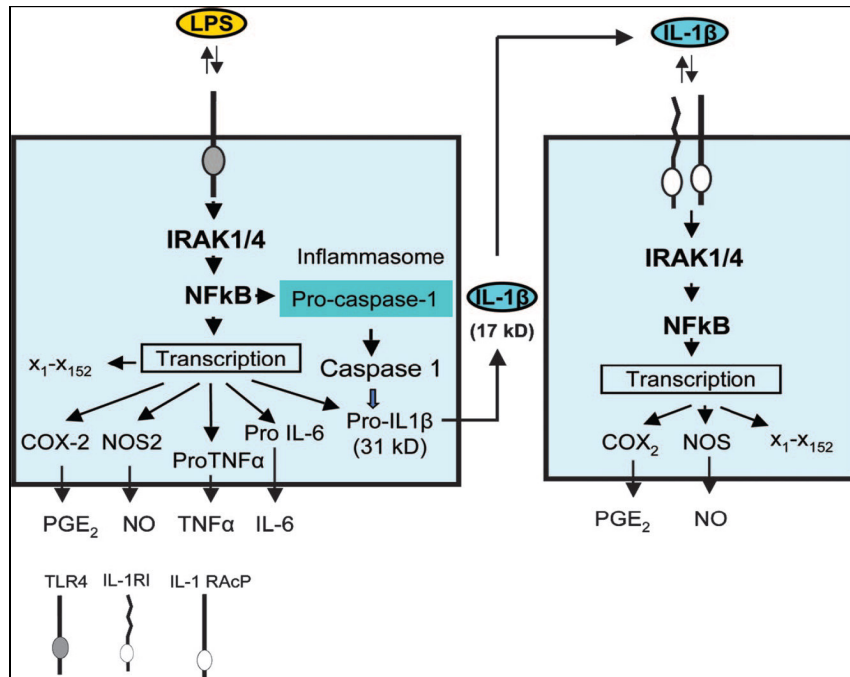
Fig. 9 Toll-like receptor, IL-1β and Caspase-1 Signaling. Taken from Sanchez-Alavez and Bartfai, 2007

IL-1β acts against various invading pathogens and helps in host defense (Johansen et al., 2007). In LPS-induced inflammation, IL-1β is a key molecule, secreted by cells of hematopoietic lineage (Brough and Rothwell, 2007). The cytoplasmic inactive precursor of IL-1β is secreted as a 31-kDa protein, lacking the secretion sequence. Activated Caspase-1 is responsible for the mature IL-1β processing by a single proteolytic cleavage. The mature, pro-inflammatory form of IL-1β is then secreted from the cells in response to pathogen interaction (Aliprantis et al., 2000).

As IL-1β is only found in the inflammatory cells just prior to release, it has been proposed that processing and release of IL-1β must be tightly linked (Fig. 9) (Brough and Rothwell, 2007).

2.5.2: During Inflammation Activated Platelets Can Produce Antimicrobial Peptides Like PPBP Which Can Kill Invading Microbes and Protect the Host Cell

The skin is an effective barrier to protect hosts against the outside environment and pathogen attack. Both resident and infiltrating immune cells can secrete antimicrobial proteins, which help in clearing bacterial and fungal infection. Platelets can play an important role in microbial defense, as bacteria-platelet interaction activates platelets and induces secretion of an array of anti-microbial peptides (Fitzgerald et al., 2006), commonly called platelet microbicidal proteins (PMPs) (Mercier et al., 2000). Microbes induce platelets to release PMPs (Mercier et al, 2000). Pro-platelet basic proteins (PPBP) are the major granular proteins in platelets and also the members of CXC chemokine family. Platelet basic protein (PBP), a derivative of PPBP, is a small ~14 KD protein that can give rise to several other derivatives, such as neutrophil-activating peptide-2 (NAPII), connective tissue-activating peptide-III (CTAPIII), thrombocidin 1 and 2 (TC1 and TC2), after post-translational modifications. Many of the derivatives of PPBP belong to the cationic anti-microbial peptide (CAMPs) family and can kill bacteria and fungi (El-Gedaily et al., 2004).

2.5.3: Role of Heme Oxygenase-1(HMOX-1/HO-1)

Heme oxygenase is a rate limiting enzyme that catalyses the degradation of heme to produce biliverdin, which is subsequently modified to bilirubin (Rydkina et al., 2002; Rushworth et al., 2005). Several stimuli, such as bacterial LPS and pro-inflammatory cytokines, can induce the production of HMOX-1. For example, rickettsial infection of cells causes oxidative damage

leading to the production of HMOX-1 as an anti-oxidative defense mechanism. Hmox-1 provides

cytoprotective effects to the injured cells through the production of bilirubin, which is an

important antioxidant (Rushworth et al., 2005).

2.5.4: IL-6 Is a Key Molecule During Acute Inflammation and Acts as "Immunological Switch" between Innate and Adaptive Immunity

IL-6 is a pleotropic cytokine that performs various functions during acute inflammation

and is elevated during inflammatory response (Gabay, 2006). IL-6 plays a dual role as both a

pro- and anti-inflammatory cytokine during pathogen interaction (Dube et al., 2004). During

chronic inflammation, IL-6 acts mostly as a pro- inflammatory cytokine (Gabay, 2006). It has

been suggested that multiple aspects of IL-6 activity are required for the transition between

innate and adaptive immunity (Fig. 10). IL-6 also acts as a switching molecule between acute

and chronic inflammation by influencing the major cell type at the site of inflammation.

Neutrophils are the major inflammatory cells during acute microbial infection and secrete

various oxygen metabolites and active enzymes to help in destroying the infected cells and

protecting the surrounding cells against injury. These secretory molecules can be toxic to the

surrounding cells if they are secreted for a prolonged duration. To protect the surrounding cells

and tissues from such damage, IL-6 promote apoptosis of the neutrophils and recruits

macrophages, which subsequently phagocytose the apoptotic neutrophils and replace the cell

population with inflammatory macrophages (Gabay, 2006). Thus, IL-6 removes the early

neutrophil population from the site of inflammation and replaces it with a sustained monocytic

response (Dube et al., 2004; Jones, 2005; Gabay, 2006). Additionally, IL-6 contributes to B-cell

differentiation, immunoglobulin secretion, and T-cell activation (Dube et al., 2004; Gabay,

2006). During intracellular infection of *Listeria monocytogenes* and *Mycobacterium*

tuberculosis, IL-6 plays a protective role (Dube et al., 2004).
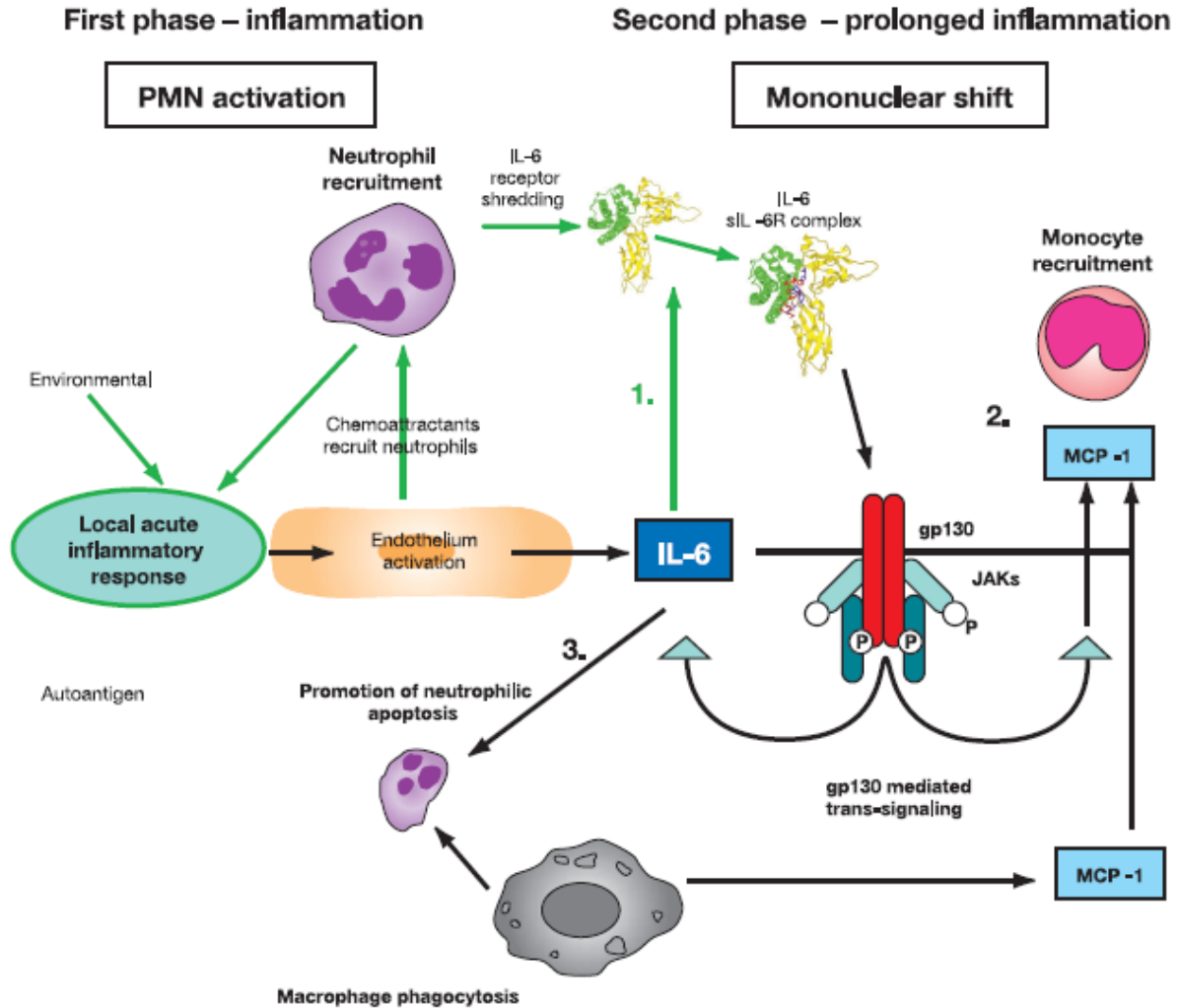
Possible role played by IL-6 in the shift from acute to chronic inflammation. Stage 1: following acute inflammatory response, IL-6 can bind with sIL-6R. Stage 2: trans-signalling through gp130 leads to monocyte recruitment. Stage 3: prolonged IL-6 leads to neutrophilic apoptosis, phagocytosis and mononuclear accumulation at the site of injury. IL, interleukin; JAK, Janus activated kinase; MCP, monocyte chemoattractant protein; PMN, polymorphonuclear neutrophil; sIL-6R, soluble IL-6 receptor.

Fig. 10 Possible Role Played by IL-6 in the Shift from Acute to Chronic Inflammation: Taken from Gabay, 2006.

2.6: Interferon-gamma Receptor1 (IFN-γR1) Is Another Membrane Receptor Which Binds to IFN-γ and Elicits Immune Response Against Invading Pathogen

IFN-γ is a type II interferon that drives the immune response towards the Th1 pathway. It is produced by T lymphocytes and natural-killer cells. It can also increase the expression of Major histo-compatibility complex I and II (MHC-I and MHC-II) on several cells, including mononuclear phagocytes, endothelial cells and epithelial cells. IFN-γ also induces the differentiation of immature myeloid precursors into mature monocytes and regulates immunoglobulin class switching on B cells (Farrar and Schreiber, 1993). IFN-γ binds to its receptors IFN-γR1 (the ligand-binding chain) and IFN-γR2 (the signal-transducing chain). Binding of IFN-γ to IFN receptors activates the JAK-STAT signaling pathway. IFN-γR1 is expressed on several cell surfaces like macrophages, monocytes, T cells, B cells, NK-cells and endothelial cells. It has been shown that various pathogens can modulate the effect of IFN-γ by down-regulating the expression of IFN-γR1 (Singhal et al., 2007; Glosli et al., 2008). The activation of IFN-γR1 makes it more sensitive to IFN-γ, so less IFN-γ is required to elicit the necessary immune response. Up-regulation of IFN-γR1 occurs due to TLR-*Chlamydia* interaction (Shirey et al., 2006), suggesting that pathogen interaction with TLRs can further modulate the expression of IFN-γR1 and subsequent binding of IFN-γ.

2.7: Host's Response to Tick Infestation

The innate immune reaction to a tick bite results in the infiltration of mononuclear cells, basophils, and eosinophils in guinepigs and predominant infiltration of neutrophils in dogs (Ferreira et al., 2003). The acquired immunity to tick bite has been studied in detail and has shown that tick infestation polarizes the immune response towards T_H2 pathway. For example,

splenocytes from *Ixodes scapularis* infested mice secreted high amount of IL-4, which is T_H2 type cytokine but reduced amount of IL-2 as well as the IFN-γ which is a T_H1 cytokine (Muller-Doblies et al., 2007).

2.8: Immunomodulatory Effect of Tick Saliva

Ixodid ticks can feed on a vertebrate host for several days to weeks. To avoid the host's immune mediated rejection, tick saliva introduces an array of pharmacologically active molecules during feeding (Schoeler and Wikel, 2001). Ticks can modulate both innate and adaptive immune response of the host (Wikel, 1996). This immune-modulation by tick saliva ensures successful blood meal acquisition for longer duration. Tick saliva can suppress the host's immune response in a variety of ways (Schoeler and Wikel, 2001). It can modulate complement activation, natural-killer cell function, antibody production, T lymphocyte proliferation, and cytokine elaboration by macrophages and T lymphocytes. It can also suppress the expression of IL-1 and TNF-α (Wikel, 1996) and inhibits the DC activation and maturation. Tick saliva also contains a wide array of immunomodulatory and anti-coagulatory compounds, which play a major role maintain blood feeding on the vertebrate host. An *in vitro* study demonstrated that the presence of tick saliva can impair the differentiation and terminal maturation of DCs in culture. Even very small amounts of diluted (≈80-fold) tick saliva (16µg/ml) can inhibit DC differentiation substantially. When DCs were cultured alone with LPS, they could up-regulate MHC class II as well as co-stimulatory molecules CD40, CD80, CD86 and CD54 on their surface. However when DCs were cultured with LPS and tick saliva, the cell surface expression of co-stimulatory molecule was abolished (Cavassani et al., 2005). To activate the T cell

response, DCs need to pass two signals, one from MHC class II and T cell receptor complex contact, and another one through co-stimulatory signal. If DCs present antigens to T cells, in absence of appropriate co-stimulation, the T cells become unresponsive to further stimulus, a state known as anergy (Janeway et al., 2001). In addition to down-regulation of co-stimulatory molecules the addition of tick saliva significantly down-regulates the expression of CCR5. This down-regulation of chemokine receptors was not unilateral since CCR1 and CCR3 were unaltered. Interestingly, tick saliva not only reduces the expression of CCR5, but also reduces the number of DCs expressing CCR5 (Oliveira et al., 2008). Thus, during infestation tick saliva can modulate the immediate immune response in skin. This synergistic immunomodulation will help *Rickettsia* to be easily disseminated while the tick is feeding on the vertebrate host for several days.

Table.1 Possible Influence of Tick Saliva in Suppression of Host's Cytokine Response (Wikel, 1996)

Cytokine	Selected biological properties of cytokine	Possible influence of cytokine suppression on host immune responses to ticks and tick-borne pathogens
Interleukin-1	Activation of T-lymphocytes in association with IL-6 and IL-2	Reduced T-lymphocyte help for antibody production and impaired delayed-type hypersensitivity and cytotoxic T-cell responses
	Costimulation of B-lymphocyte differentiation and proliferation by induction of IL-6 and synergy with IL-4	Reduced antibody responses
	Nonspecific resistance to infection	Increased susceptibility to infection
Tumor necrosis factor-α	B- and T-lymphocyte activation	Reduced antibody and cell-mediated immune responses
	Nonspecific resistance to infection	Increased susceptibility to infection
Interleukin-2	Autocrine stimulation of T-lymphocyte growth	Reduced T-lymphocyte helper and effector functions (delayed-type hypersensitivity and cytotoxic T-cell responses)
	Augmentation of B-lymphocyte growth and immunoglobulin production	Reduced antibody responses
Interferon-γ	Induction of class I and II MHC	Reduced ability to present antigens requiring class II MHC and ability of cytotoxic T-cells to bind class I MHC essential for killing
	B-lymphocyte differentiation and immunoglobulin production	Reduced antibody responses
	Enhanced macrophage killing	Reduced ability of macrophages to phagocytize and kill
	Promotes NK cell cytotoxicity	Reduced NK cell activation
	Anti-viral properties	Reduced resistance to viruses

Chapter3: Materials and Methods

3.1 Solutions

Media for cell culture

10% FBS/DMEM solution was made by adding 25 ml of FBS (Fetal Bovine Serum, Collected in Central America. Catalogue no. SV30014.03) to 225 ml of DMEM (Dulbecco's Modified Eagle Medium 1X, 4.5 g/l D-glucose, L-Glutamine, Sodium pyruvate (GIBCO, Invitrogen). The solution was mixed completely and stored at 4°C.

Phosphate buffer saline (PBS):

The working concentration for PBS was 0.137mM NaCl/2.7mM KCL/8mM Na_2HPO_4. To prepare this solution, 160 g of NaCl, 40 g of KCl, 28.8g $Na_2HPO_4.2H_2O$ and 4.8 g of KH_2PO4 were added to 1.97 L of Milli-Q™ H_2O. The pH was adjusted to 7.5 with approximately 10-15 ml of 5N NaOH. Milli-Q™ water was added to make the volume 2 L, and the solution was stirred until components mixed completely and stored at room temperature.

Solutions for cytospin

Rickettsiae were stained using Diff-Quick Stain set, (Dade Behring, catalog no. B4132-1A F4710/72) according to the manufacturer instructions.

Rickettsia staining dye to check viability

LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, catalog no. L7012)

0.15 µl of Component A: SYTO 9 dye in DMSO

0.15 µl Component B: Propidium Iodide in DMSO

Component C: BacLight mounting oil for bacteria immobilized on membranes

3.2 Vero Cell Stock Culture

Vero cell stocks (ATCC no.CCL-81) were obtained from the Centralized Cell Culture Laboratory at the Louisiana State University, School of Veterinary Medicine.

3.3 Mouse Srains

BALB/C mice were purchased from the Division of Laboratory and Small Animal Medicine (DLAM), LSU, Veterinary Medicine Building and were maintained in animal care facilities in the Veterinary Medicine building. All animal experiments were carried out under the guidance of Louisiana State University's Animal Care and Use Committee and the appropriate guidelines set forth by the National Institutes of Health.

3.4 *Amblyomma americanum* Tick Colonies

Uninfected and *Rickettsia amblyommii*-infected unfed tick colonies were obtained from a colony maintained by the Tick Research Lab, Department of Entomology, Texas A&M University (College Station, TX), which was founded with specimens originally collected in Sutton County, TX. The original colony of ticks is maintained without regular introduction of wild-caught ticks.

3.5 *Rickettsia amblyommii* Stocks

The *R. amblyommii* (Darkwater strain) stock was a generous gift from Dr. Christopher D. Paddock, Infectious Disease Pathology Branch, Centers for Disease Control and Prevention, Atlanta, GA.

3.6 Vero Cell Culture

Frozen Vero cells (P7) were taken out of liquid nitrogen and allowed to thaw at 37°C for 5 minutes. The cells were collected with a pipette from the tube and added to 10 ml of complete fresh media. pelleted at 150 x g for 5 minutes, the old media was discarded and the cell pellet was re-suspended in 5 ml fresh media, transferred to a T25 tissue culture flask, and incubated at 34°C. All experiments were carried out in a Biological Safety Cabinet, class II, Type A2, using sterile technique.

3.7 Splitting Uninfected Vero Cells

When the cells were confluent, the media was removed and the cells were rinsed with 2 ml of 0.25% Trypsin with EDTA. The flask was incubated at 34°C for 5 minutes to allow detachment of the cells which then were suspended in 4 ml of fresh media. Next, 2 ml of this mixture were added to a T75 flask along with 10 ml fresh media and the cells were incubated at 34°C for 2-3 days until the cells formed an adherent monolayer.

3.8 Infecting the Uninfected Vero Cell Culture

Frozen *Rickettsia amblyommii* infected cells (P4) were taken out from a -80°C freezer and. The thawed cells were added to the uninfected Vero cell flask and incubated for an hour at 37°C. The

media was removed and replaced with fresh media. The flasks were incubated at 34°C for 2-3 days until the cell monolayer was infected.

3.9 Checking Infection by Cytospin

Infected flasks were taken out of the incubator and the inside corner of the flask was scratched with the tip of a pipette. The scratched cells were suspended in the media and 300 µl of this cell-media mixture was removed. Next, 100 µl of this was added to the top chamber of the Cytospin® column and 200 µl to the bottom chamber. A dual-etched glass slide was placed at the end of the spin column and the cells were spun in the Cytospin® centrifuge for 10 minutes at 1000 x g.

After spinning the cells, the slide was removed from the chamber and allowed to air-dry. The slides were then fixed and stained using the Diff-Quick stain set. The slides were again air-dried and observed under the microscope for intracellular infection (100X). Live bacteria, if present, appeared as rod-shaped purple structures located inside the cells.

After the cells were infected the flasks were scraped to detach the cell layer. The cell suspension was lysed using a 5cc syringe attached to 27 gauge needle using 10 repetitions of the syringe uptake-expulsion cycle. The lysed cells were transferred to a centrifuge tube and centrifuged for 10 minutes at 275 x g at 4°C, and then filtered with a sterile 2 micron filter. 100 µl of the cell suspension was removed and transferred to a 1.5 ml tube, and cells were peletted at 1300 rpm at 4°C for 10 minutes to harvest the *Rickettsia* pellet. After discarding the supernatant, the pellet was re-suspended in 500 µl of 0.85% NaCl solution and spun at 13,000 x g at 4°C for 10 minutes. The suspension was diluted with 0.85% NaCl solution to 1:30, and 100 µl of this cell

suspension was mixed with 0.3 µl of dye mixture (LIVE/DEAD kit, Solution A: Solution B=1:1) and incubated at room temperature in the dark for 15 minutes. 10 µl of the stained cell suspension was transferred to a bacterial counting chamber and observed under the microscope. Bacterial numbers (*Rickettsia*/ml) were recorded using the following formula: Rickettsial concentration = *Rickettsia* in 5 chambers x 5 x 0.05 x 10^6 x dilution factor.

3.10 Infestation of Mice with *Amblyomma americanum* Ticks

Infestation was done according to procedures described by Schoeler et al (1999). Briefly, BALB/C mice (male, 4-6 weeks of age) were harnessed with two 1.5 ml centrifuge tubes, cut in half and attached on both sides of their body as capsules with a 4/1 (w/w) mixture of calphonium (rosin) and beeswax. Mice were infested with ten tick nymphs (Infected/Uninfected) on either side. Empty capsules were used for the control group of mice. Nymphs were allowed to feed until fully engorged (5-7 days). After infestation, capsules were removed and mice were euthanized. Tissues were collected, snap frozen in liquid nitrogen, and then stored at -80°C for future use.

3.11 Intradermal Inoculation of Mice with *R. amblyommii*

Mice were shaved as before and 1×10^5 *Rickettsia*/ml in 50 µl solution was injected on each side. Capsules were added on each side on the inoculation site to maintain similarity with the tick infestation experiment. PBS was injected instead of bacterial culture in the control group. 7 days after infection, capsules were removed and the mice were euthanized. Tissues were collected, snap frozen in liquid nitrogen and then stored in -80°C for future use.

3.12 RNA Processing for Real-Time PCR

Isolation of RNA: Frozen skin tissues were brought to the workbench from the -80°C freezer in liquid nitrogen to avoid thawing of the samples. To grind the skin tissue into powder with minimal RNA loss, mortars and pestles were pre-chilled by pouring liquid nitrogen on them at least three times. The skin tissues were transferred from the liquid nitrogen container to the liquid nitrogen in the mortar. After waiting a few seconds the tissue was broken into small pieces using the pestle. Liquid nitrogen was poured to the mortar, and after a few seconds when the liquid nitrogen was almost evaporated, the tissues were very quickly ground into a semi-powdery form. This was repeated 2-3 times to convert the tissue completely into a powdery form. When the grinding was complete, mortars were kept at room temperature for approximately 10 minutes until water droplets were seen to form outside the mortars. Then, using a 1000 µl pipette, 1 ml Trizol (Invitrogen) was added to the mortars and the tissues were homogenized for another 30 seconds with the pestle. The homogenate was transferred into 1.5 ml tubes and kept at room temperature for 2-3 minutes for the complete dissociation of the nucleic acids. 200 µl of chloroform was added in each tube and the tubes were vigorously vortexed for 30 seconds. Samples were kept at room temperature for another 2-3 minutes. Samples were then centrifuged at 10,000 x g for 15 minutes at 4°C in an Eppendorf table-top centrifuge to separate the organic phase from the RNA. The clear aqueous phase was transferred to another 1.5 ml tube and the RNA was precipitated by adding 600 µl of isopropanol to the tubes and mixing by pipetting up and down. Samples were incubated at room temperature for two hours and the RNA was peletted at 10,000 x g for 10minutes at 4°C. The supernatant was discarded and 1 ml ethanol was added to each tube, which were vortexed for few seconds to dislodge and wash the pellet. The RNA

was repelleted at 10,000 x g for 5 minutes. The supernatant was discarded and pellets were air dried under the hood for 5-10 minutes. Subsequently, the pellet was dissolved in 50 µl of DNase-RNase-free water, incubated for 10 minutes on a dry heat block at 50°C, and finally vortexed, centrifuged briefly, and stored at -80°C.

DNase Treatment: Extracted RNA was purified using Ambion DNase I enzyme and buffer to remove any residual DNA from the extracted RNA. A master mix was prepared by combining 65 µl of RNase-free water, 10 µl DNase buffer and 15 µl (30 units) of DNaseI enzyme for each sample. 90 µl of this master mix was added to 10 µg of RNA sample (in a volume of 10 µl) in a 1.5 µl centrifuge tube (total volume 100 µl). The master mix was mixed by pipetting up and down, briefly centrifuged and incubated for 30 minutes at 37°C. RNA was recovered using a Zymo RNA Clean-up kit (Zymo Research). 400 µl of RNA binding buffer was added to the tube, mixed thoroughly by pipetting up and down and was transferred to a Zymo-spin column. The column was centrifuged for 30 seconds at high speed (<10,000 x g). RNA was washed twice using the RNA wash buffer and again centrifuged at high speed (<10,000 x g) for 30 seconds. Finally RNA was eluted by adding 25 µl of RNase-free elution buffer to the column and centrifuging at 12,000xg for 10 seconds. Eluted RNA was stored at -80°C for downstream use.

Reverse Transcription: 15 µl of master mix was prepared using the iScript cDNA Synthesis Kit (BioRad) by adding 10 µl nuclease-free double distilled water, 4 µl 5X iScript Reaction mix, and 1 µl of reverse transcriptase (iScript RT) for each sample. This master mix was added to approximately 1µg of RNA sample in a 0.5ml tube. 1 µl of water was added instead of the RNA sample in the no-RT control tubes. Positive control was used by serially diluting RNA sample in

1:5, 1:25, and 1:125 dilutions. Tubes were placed in a MJ Research PTC Thermal Cycler and run under the following conditions: 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and 20°C hold. After completion, 60 µl of nuclease free water was added to the cDNA sample, vortexed, centrifuged and stored in -20°C until further use.

3.13 SuperArray Real-Time PCR

SuperArray PCR analysis was performed using the Mouse Innate and adaptive immune response Pathway PCR Array kit (PAMM-052) from SABiosciences.

RT^2 First Strand Synthesis: SABiosciences SuperArray RT^2 First Strand kit (Cat. No. C-03) was used to perform the first strand cDNA synthesis reaction. 10 µl of RT cocktail was added to 10 µl of genomic DNA elimination mixture, mixed gently by pipetting up and down and incubated at 42°C for 15 minutes. Next, this mixture was heated at 95°C for 5 minutes to degrade the RNA and to inactivate the reverse transcriptase. After this, 91 µl of ddH_2O was added to each 20 µl of cDNA synthesis reaction and mixed well and the tube was kept on ice until the next step or stored at -20°C until further use.

SuperArray Real-Time PCR: A PCR cocktail was prepared by mixing 1275 µl of 2X SuperArray RT^2 qPCR master mix, 102 µl of diluted first strand cDNA synthesis reaction and 1173 µl ddH_2O to make a final volume of 2550 µl. 25µl of the experimental cocktail was added to each well of the PCR array 96 well array plate with a multi-channel pipette. The plate was vortexed for 30 seconds and then centrifuged for 15 minutes at 1500xg at 4°C. For each sample, 10 µl of the first-strand cDNA synthesis cocktail was added to appropriate wells of the 384-well PCR array plate with a multi-channel pipette. The plate was covered with a DNase-RNase free film

and centrifuged for 15 minutes at 1500 rpm at 4°C. Reactions were carried out on an Applied

Biosystems 7900 real-time thermocycler. The data was analyzed with the SDS 2.2.2 software

(Applied Biosystems). Gene expression was quantified using the cycle number at which each

sample reached a fixed fluorescence threshold (C_T) during the annealing step.

3.14 Real-Time PCR

For Quantitative Real-time PCR analysis, primers were designed by using the Primer3

web utility (http://frodo.wi.mit.edu/) and confirmed by blasting the primer pairs against the

National Center for Biotechnology Information (NCBI: http://blast.ncbi.nlm.nih.gov/Blast.cgi)

website. Primers were diluted to working concentration (100 µM) before performing real-time

PCR (Table 2). A master mix was prepared using iTaq SYBR Green Supermix with Rox (Bio-

Rad, catalog no. 172-5850) by combining 17.5 µl 2x iTaq Green Supermix with Rox, 1.75 µl of

forward and reverse primers (1.8 µM final concentration) and 9 µl RNase free water for a total

volume of 30 µl per sample. Samples with no reverse transcriptase (RT) were run to check for

genomic DNA contamination. 5 µl (10 ng) of appropriate cDNA sample and 30 µl of master mix

was added to each well of a 96-well plate. Water was used as a no-DNA control. The 96-well

plate was always kept on a cooler plate. After adding the template and master-mix, the plate was

covered with a DNase-RNase free film, vortexed for 30 seconds, and centrifuged for 5 minutes at

1500 x g at 4°C. The film cover was removed and the samples were transferred to a 384-well

plate (Applied Biosystem) in triplicate using a Matrix electronic repeating pipette (Matrix

technologies). Plates were again covered with a DNase-RNase free film cover, and spun at

1500xg for 5 minutes at 4°C. Reactions were carried out on an Applied Biosystems 7900 real-

time thermocycler and data was analyzed with the SDS 2.2.2 software packages (Applied Biosystems). Gene expression was quantified using the cycle number at which each sample reached a fixed fluorescence threshold (C_T). Samples without RT were either unamplified or had at least 1,000-fold lower expression than the actual samples. *Gapdh* was used as a house keeping gene and to control variations in RNA concentrations between samples. Data were represented as the percent difference in C_T value (log_2) of each sample subtracted from *Gapdh* CT value ($\Delta C_T = C_T$ *Gapdh*- C_T gene of interest).

3.15 Statistical Analysis

The GraphPad Prism Software (GraphPad Software, San Diego, CA) was used to statistically analyze the data.

Table.2 List of Primers Used in this Study

Genes	Forward primer	Reverse primer
Gapdh	TGCACCACCAACTGCTTAGC	TGGATGCAGGGATTCACT
TLR2	CTCCCACTTCAGGCTCTTTG	GCCACTCCAGGTAGGTCTTG
TLR4	GGCAGCAGGTGGAATTGTAT	AGGATTCGAGGCTTTTCCAT
TLR9	ACTTCGTCCACCTGTCCAAC	TCATGTGGCAAGAGAAGTGC
MYD88	CATGGTGGTGGTTGTTTCTG	CTGTTGGACACCTGGAGACA
TRAF6	GCCCAGGCTGTTCATAATGT	CGGATCTGATGGTCCTGTCT
MAPK8	CGGAACACCTTGTCCTGAAT	GAGTCAGCTGGGAAAAGCAC
TOLLIP	CCACTGTCTGGCTAGGCTTC	AGCAGTGAGGGCTAATGTGG
IFNGR1	TCCTCCACCCTGATCATCTC	AGACTTACGGCTGGCTTTGA
CLEC7A	GGAATCCTGTGCTTTGTGGT	ATTCTGTGGGCTTGTGGTTC
CD80	CCTTGCCGTTACAACTCTCC	CAGGCCCAGGATGATAAGAG
CD86	CTCCTCCTTGTGATGCTGCT	GCCTTCACTCTGCATTTGGT
CD207	GGTCGTGTGGACAACATCAG	TGCATCTGAACCTCAGCATC
CCR1	TCAAAGCATGACCAGCATCTA	CTTGTAGTCAATCCAGAAAGGTAAA
CCR5	CGAAAACACATGGTCAAACG	GTTCTCCTGTGGATCGGGTA
CASP-1	CACAGCTCTGGAGATGGTGA	GGTCCCACATATTCCCTCCT
IL-1β	CCCAAGCAATACCCAAAGAA	GCTTGTGCTCTGCTTGTGAG
HMOX-1	TGCTCGAATGAACACTCTGG	TCCTCTGTCAGCATCACCTG
PPBP	CATCCCTTCCCCTTTCATTT	TCCGTTGCAATTGACCACTA
LY96	CCAATGGATTTGTGCATGTT	GGCACAGAACTTCCTTACGC
PGLYRP3	GCCACAGTGATGTGTCCAAC	CGACGGGACTGTAGCATTTT
LBP	GGCTGCTGAATCTCTTCCAC	TAAGGCTGCAGGTCAGAGGT
IL-6	CCGGAGAGGAGACTTCACAG	TCCACGATTTCCCAGAGAAC
TREM-1	TTCACAGGAGGAGCTGAGGT	GAGCACATCCCCAAGATGAT

Chapter 4: Results

4.1: Expression of Several Innate and Adaptive Immune Response Genes in Skin During Rickettsial Infection via Intradermal Inoculation As Well As Tick Transmission

To investigate how tick transmission impacts the host immune response to *Rickettsia* infection, we compared the mRNA expression of a number of host-response genes in skin tissue of mice infected with *Rickettsia* by either intradermal inoculation or by tick transmission. For gene analysis we utilized real-time PCR arrays to examine the mRNA expression of 84 different immune response genes. Skin tissue from 3-5 mice inoculated with 1×10^5 *Rickettsia*/ml by intradermal (ID) inoculation were compared to skin tissue from 3-5 mice infested with *Rickettsia* infected ticks. A time point of 5-7 days post inoculation/infestation was utilized to allow the transmission of bacteria from the tick and the development of an immune response to *Rickettsia* infection. Interestingly, there was a significant difference in mRNA expression of a number of immune response genes. mRNA expression for several inflammatory genes were higher in the tick-infested mice compared to ID inoculated mice, including genes whose products play important roles in pathogen recognition (Fig. 11, right side of graph). An additional group of genes had significantly lower mRNA expression in the tick infested mice compared to the ID inoculated mice (Fig. 11, left side of graph). Several of the genes with increased mRNA expression following tick transmission included: cytokines *Ccl2, Il1b, Il6* and *Il10*; inducible nitric oxide synthetase, *Nos2*, as well as the stress response gene, *Hmox1* (Fig. 11). mRNA for these genes are often transcribed following immune cell activation. Several PRR-related genes also had higher levels of mRNA with tick transmission of *Rickettsia* compared to ID inoculation. This included TLRs (*Tlr1, Tlr4*) as well as a C-lectin receptor (*Clec7a*) and genes whose

products are involved in binding of LPS to TLRs (*Lbp, Ly96*) (Fig. 11). This suggests that tick transmission may increase the expression of several host response proteins necessary for the detection of *Rickettsia* infection. However, mRNA expression of several genes involved in the signal transduction of PRRs including *Ikbkb*, *Irak1*, *Irak2*, *Mapk8*, *Traf6* and *Tollip* had significantly lower levels of mRNA with tick transmission of *Rickettsia* compared to ID inoculation (Fig. 11). Decreased mRNA and subsequent protein production of these molecules could inhibit the activation of cells following PRR stimulation. Other than the genes responsible for inflammation, we studied the mRNA expression of several other chemokines including *Ccr1, Ccr5* responsible for LC migration to skin during inflammation, and the co-stimulatory molecules those are expressed during LC maturation including *Cd80* and *Cd86*. There was a general trend for upregulation of the mRNAs of all the four genes in the tick infested group, again suggesting a role played by tick saliva during infestation.
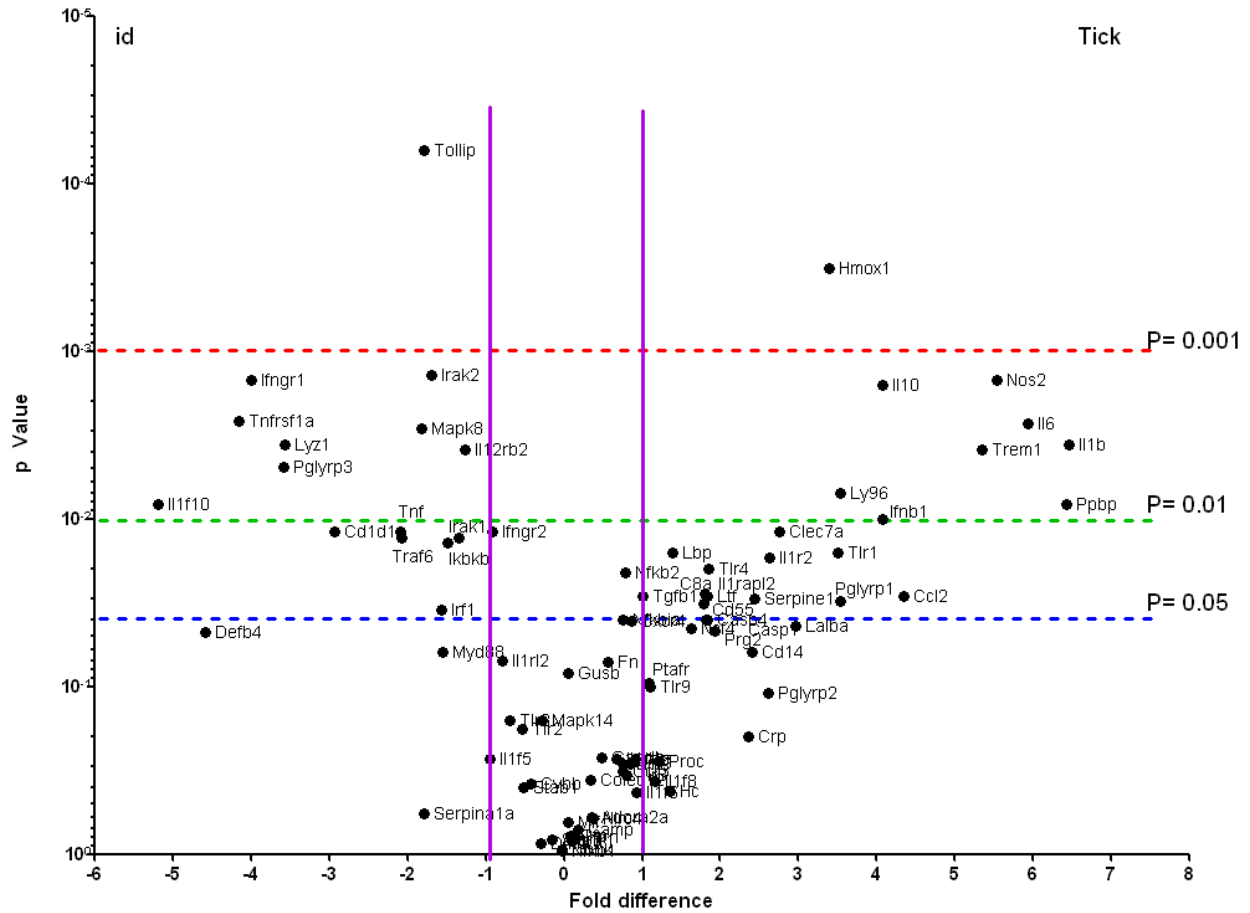
Figure. 11 mRNA expression of innate and adaptive immune response genes using SABioscience SuperArray. For ID inoculation, mice were inoculated with 50µl of *Rickettsia amblyommii*. Seven days following ID inoculation, skin tissue at the site of inoculation/infestation was removed and processed for RNA isolation. cDNA was generated from RNA and analyzed by quantitative real-time PCR analysis. Data is presented as a volcano plot, with the fold difference in gene expression in tissue samples from *Rickettsia* inoculated id and *Rickettsia* transmitted via tick on the X axis and the P value of a t-test for the difference between the groups (three to five mice per group).
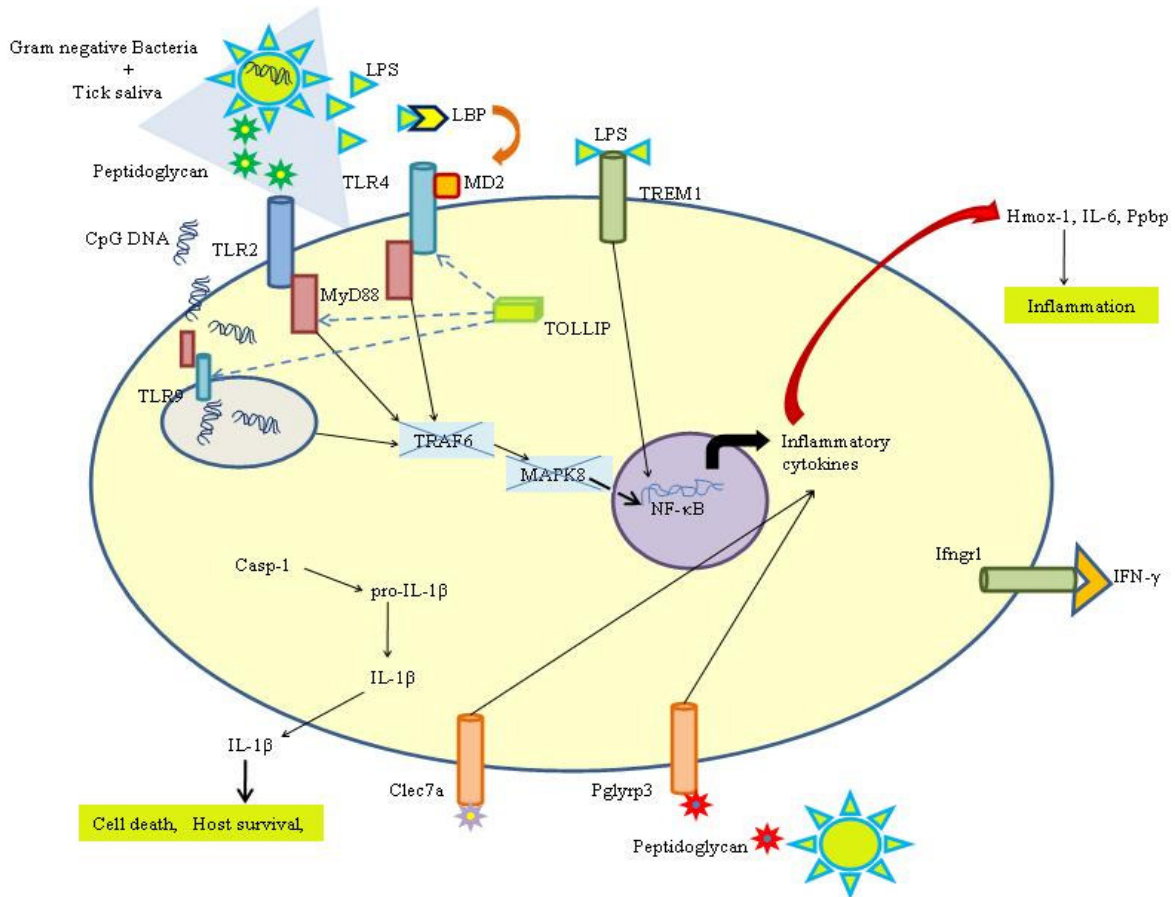
Fig. 12 Diagram showing the possible role of pattern recognition receptors (PRRs), their adaptor molecules and other innate immune genes during rickettsial infection and tick bite: PAMP (LPS, Peptidoglycan, CpG DNA) recognition by the PRRs (TLR2, TLR4, TLR9, TREM-1 and CLEC7A) and other cell surface receptors like IFNGR1 leads to the activation of the adaptor molecules. The activation of these adaptor molecules in turn activates the downstream signaling molecule leading to the activation of transcription factor NF-κB. The activated NF-κB can transcribe several cytokines as well as stress-induced genes which can cause inflammation.

Role of Langerhans Cell in Innate and Adaptive Immunity
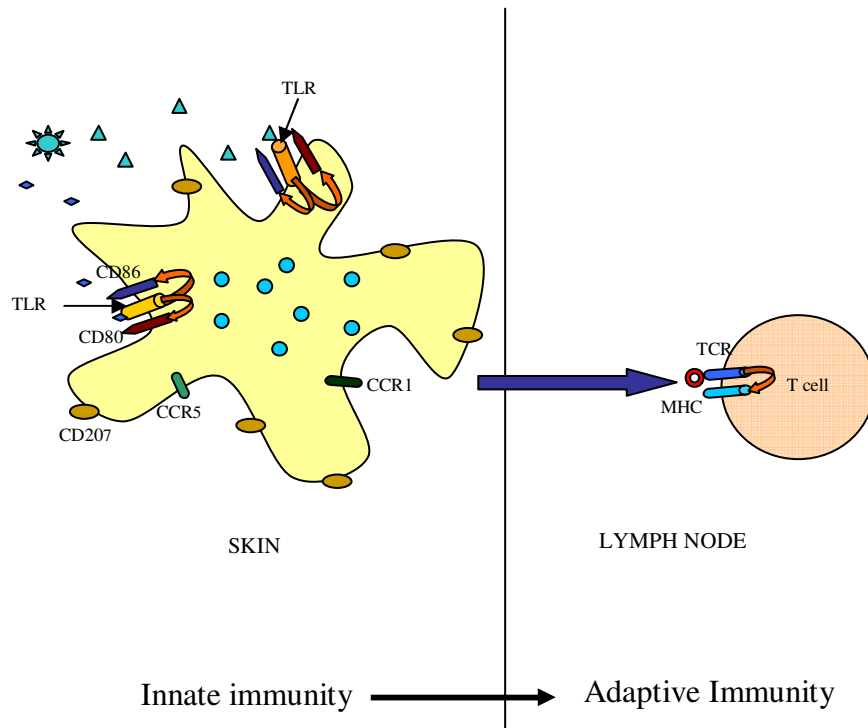
Fig. 13 Bridge between the innate and adaptive immune system by Langerhans cells. LCs are the major antigen presenting cells in skin. During microbial infection immature LCs can up-regulate several chemokine receptors like CCR1 and CCR5 and migrate to the site of infection, (*i.e.* skin), where they capture several microbial components and up-regulate another set of chemokine receptors like CCR7 and migrate from skin to the draining lymph node to present the antigen to the T-cells.

4.2: Expression of the PRRs and Their Adaptor Molecules During Tick Bite and Rickettsial Infection

4.2.1: mRNA Expressions of Toll-like Receptor 2, 4 and 9 (*Tlr2, 4, 9*), the Major PRRs in Gram Negative Bacterial Recognition
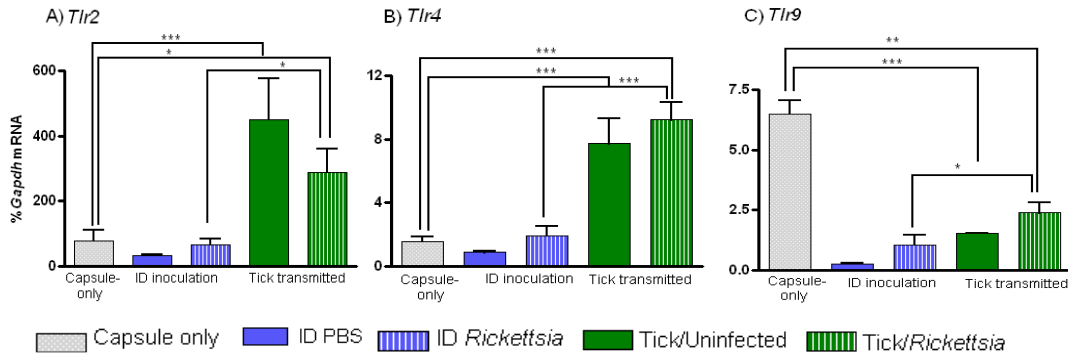
Figure. 14 mRNA expression of Toll-like receptors in skin [(A) *Tlr2*, (B) *Tlr4*, (C) *Tlr9*]. Mice were either infested with infected ticks, uninfected ticks or needle inoculated with *Rickettsia*. Microcentrifuge capsules were placed on all mice for tick containment and at the ID inoculation sites and mice with capsules only and PBS injection were used as control groups. Mice were infected/infested for 5-7 days, euthanized and skin tissues were collected. RNA was extracted and processed for quantitative real time PCR analysis and values were calculated relative to expression of *Gapdh* controls. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$

To study the expression of immune response genes in detail that were either upregulated or downregulated in the SuperArray data, gene-specific quantitative real-time PCR was performed for each gene of interest (Table 2). Transcription of *Tlr4* and *Tlr2* was upregulated in the tick transmitted group compared to the ID inoculated *Rickettsia* group (Fig. 14). Analysis of all groups indicated that infected and uninfected tick infestation groups had higher expression of both the *Tlr* genes compared to the ID inoculated *Rickettsia* group, suggesting that ID inoculation of *Rickettsia* did not induce expression of *Tlr2* and *Tlr4*. As such, it may be that tick saliva rather than bacterial infection can induce the expression of *Tlr2* and *Tlr4* mRNA.

In contrast, *Tlr9* mRNA expression was decreased in both the tick infested groups compared to the capsule only tissues. Between the experimental groups, *Tlr9* mRNA expression was higher in the tick transmitted *Rickettsia* group compared to the ID inoculated *Rickettsia* group, although by a small difference ($p < 0.05$). Thus, mRNA expression for *Tlr2*, *Tlr4* and *Tlr9*, three TLRs involved in bacterial recognition, were higher in skin tissues of tick-transmitted *Rickettsia* than ID transmitted *Rickettsia*.

4.2.2: Expression of the Adaptor Molecules Involved in Toll Signaling (MYD88, TRAF6, MAPK8, TOLLIP)

4.2.2.1: *Myd88* mRNA was Upregulated in the Tick Transmitted Group Compared to the *Rickettsia* Infected Group

Figure. 15 mRNA expression of the adaptor molecule *Myd88* involved in Toll signaling in skin. Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.00$

Activation of TLRs after pathogen recognition requires the recruitment of the adaptor protein MYD88 to induce the signal to activate the MAP kinases and transcription factor NF-κB (Dunne and O'Neill, 2003). Data analysis showed significantly higher *Myd88* mRNA expression in the tick transmitted groups than the ID *Rickettsia* infected group (Fig. 15).

4.2.2.2: Expression of *Traf6* and *Mapk8* mRNA was Unaltered in All the Groups

Figure. 16 mRNA expression of the adaptor molecule *Traf6* involved in Toll signaling in skin. Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *p <0.05; **p <0.01; and ***p <0.001
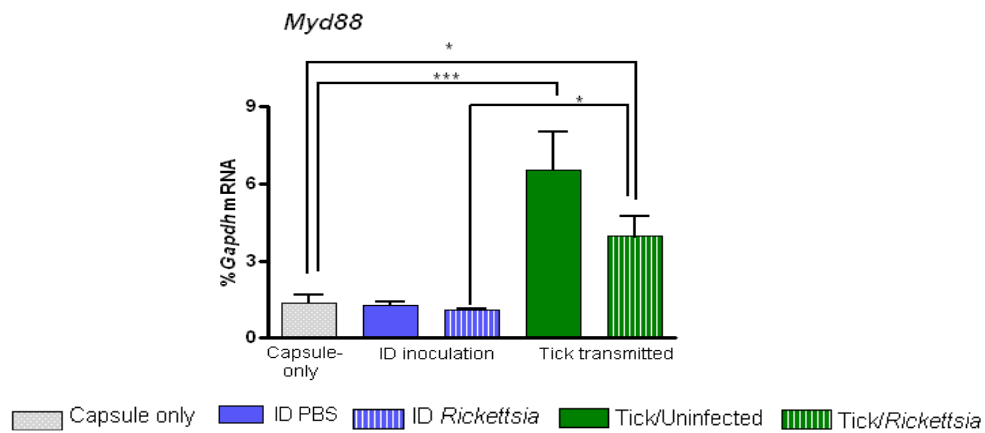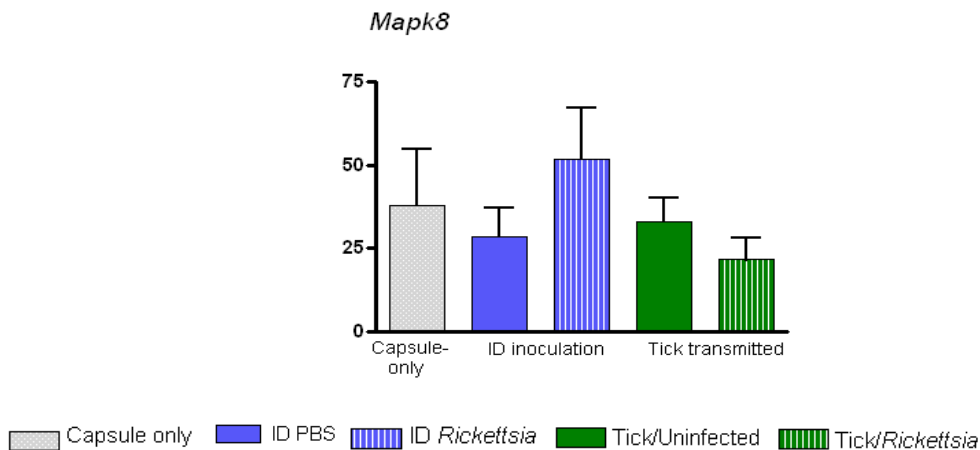

Figure. 17 mRNA expression of the adaptor molecule *Mapk8* involved in Toll signaling in skin. Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *p <0.05; **p <0.01; and ***p <0.001

The activation of NF-κB in the TLR pathway also requires two other molecules, TRAF6 and MAPK8, which act as signaling mediators in the toll signaling pathway. The activation of TRAF6 subsequently leads to the activation of several other downstream signaling molecules and eventually activation of MAPK8 (also known as JNK), which is a member of the MAP

kinase family (Armant and Fenton, 2002; Akira, 2003). In the data, the expression of both *Traf6*

and *Mapk8* mRNA was unaltered (Fig. 16 and 17). Thus, although mRNA expression of *Tlr2*,

Tlr4 and *Myd88* were significantly increased in the tick infested group, not all components of the

signaling pathway have increased mRNA.

4.2.2.3: mRNA Expression of *Tollip* which is a Negative Regulator of Toll Signaling was Also Unaltered in All the Groups

TOLLIP acts as a negative regulator of the toll signaling pathway and is believed to be

responsible for IL-1R/TLR signaling termination (Didierlaurent et al., 2006). SuperArray

analysis demonstrated a substantial difference in gene expression between ID inoculated

Rickettsia and tick-transmitted *Rickettsia*, although, by quantitative Real-Time PCR analysis,

neither tick infestation nor rickettsial infection had any effect on *Tollip* mRNA expression (Fig.
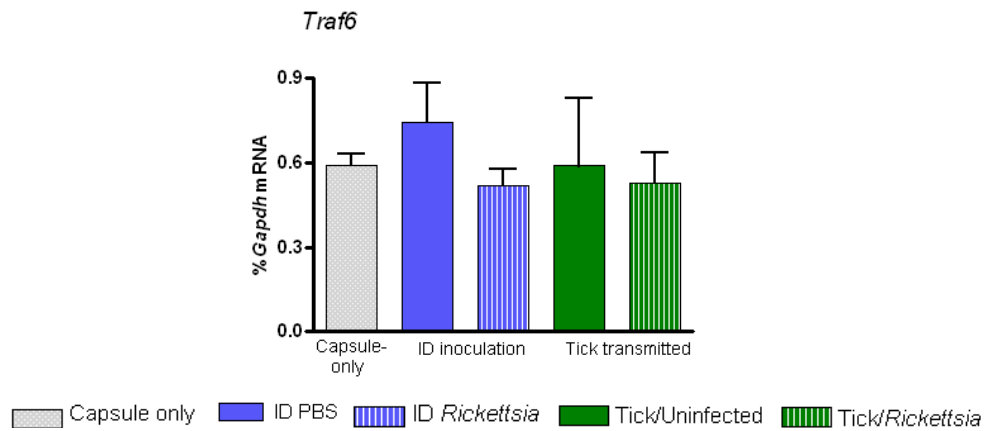
18).
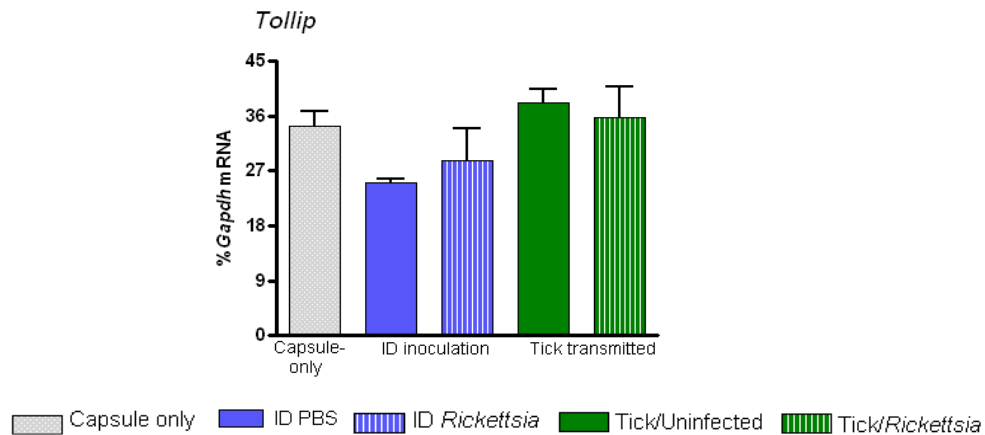
Figure. 18 mRNA expression of the adaptor molecule *Tollip* involved in Toll signaling in skin. Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$

4.2.2.4: Expression of LPS Binding Protein (*Lbp*) and *Md2* mRNA was Highly Upregulated in the Infected Tick Group
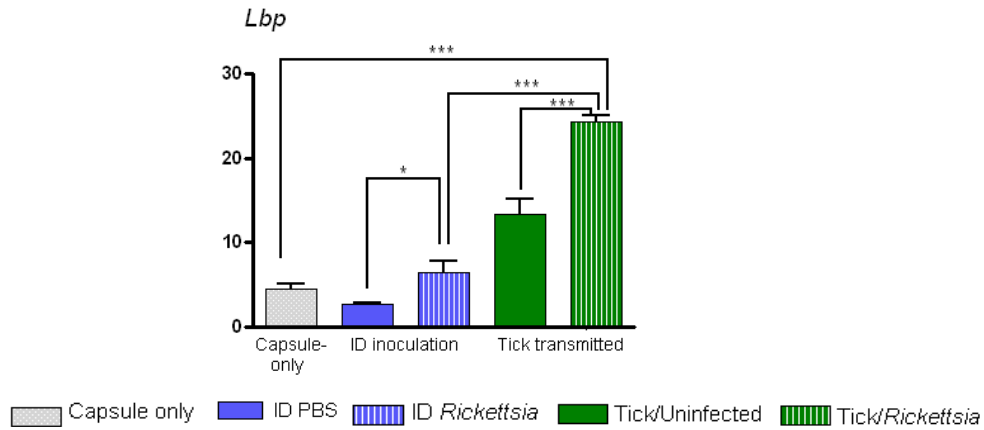
Figure. 19 mRNA expression of *Lbp* involved in LPS recognition in Toll signaling pathway in skin. Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$
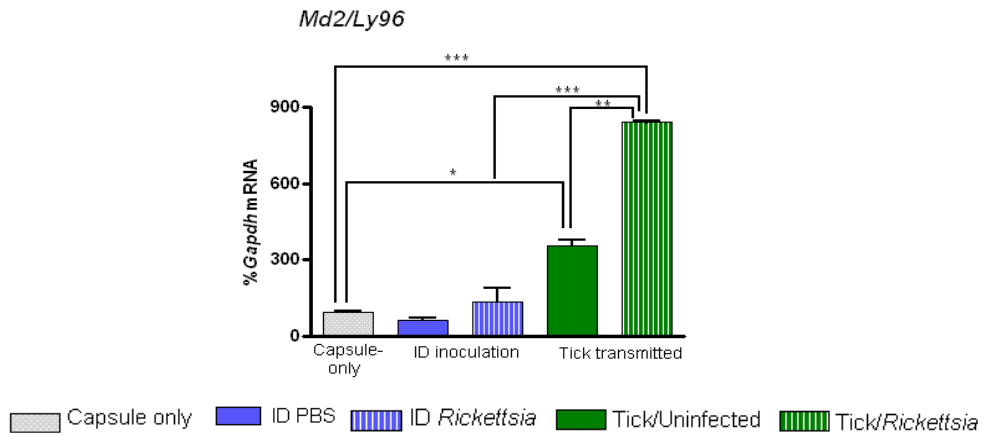
Figure. 20 mRNA expression of *Md2/Ly96* involved in the delivery of LPS to the TLRs in skin. Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$

During bacterial infection, LBP helps to extract the LPS from Gram-negative bacterial cell walls and delivers them to the TLR4-Ly96/MD2 complex. Figures 19 and 20 demonstrate that mice infested with *Rickettsia*-infected ticks had significantly high mRNA expression of both *Lbp* and *Ly96/Md2* compared to the needle inoculated *Rickettsia* group and uninfected tick

group. As both of the adaptor molecules help in the efficient delivery of the bacterial LPS, this suggests that during infected tick infestation, these two molecules are upregulated for better bacterial recognition and subsequent delivery of LPS to the TLRs molecule to activate the TLR pathway via TLR2 and TLR4.

4.3: mRNA Expression of the Non-TLR PRRs *Trem-1*, *Clec7a* and *Pglyrp3*

TREM-1, PGLYRP3 and CLEC7A are PRRs other than TLRs. All three PRRs recognize different components of bacteria. TREM-1 is a PRR belonging to the immunoglobulin superfamily that recognizes several components of bacteria (Bleharski et al., 2003; Colonna and Facchetti, 2003). CLEC7A, a C-type lectin receptor acts as an antigen binding molecule during host response to pathogen interaction (Willcocks et al., 2006). PGLYRP3 is the molecule responsible for bacterial peptidoglycan recognition (Liu et al., 2001).

Figure. 21 mRNA expression of *Trem-1*(A), *Pglyrp3* (B) and *Clec7a* (C). Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *p <0.05; **p <0.01; and ***p <0.001

Similar to the *Tlr2* and *Tlr4* mRNA expression, *Trem-1* (Fig. 21A) and *Clec7a* (Fig. 21B) mRNA expression was also increased with tick infestation. The increased mRNA expression of these genes may indicate the recruitment of dendritic cells to the site of the tick infestation or the

activation of dendritic cells or endothelial cells at the site of tick bite. *Trem-1* mRNA was also

increased by *Rickettsia* infection in association with tick transmission (Fig. 21A). This result

indicates that *Trem-1* mRNA is upregulated only in response to *Rickettsia* when transmitted via

tick saliva. Increased *Trem-1* mRNA expression in addition to TLR expression may increase the

anti-bacterial responses of cells that express both TREM-1 and TLRs, including neutrophils and

monocytes. The inflammatory reaction is more robust in cells expressing these receptors (*i.e.*,

TLRs and TREM-1) than in the cells expressing only TLRs, like endothelial cells. In contrast

Pglyrp3 mRNA was significantly upregulated in the needle inoculated *Rickettsia* group

compared to the tick infestation groups. Tick infestation suppressed the *Rickettsia*-induced

Pglyrp3 mRNA expression (Fig. 21C).

4.4: Recognition of Bacterial Components by PRRs Leads to the Production of Several Innate Immune Genes which Help to Protect the Host Cell and the Host from the Infection

4.4.1: Expression of *Casp-1* mRNA Was Upregulated in the Uninfected Tick Group Compared to the *Rickettsia* Infected Group, Whereas *IL-1β* mRNA Was Upregulated in the Infected Tick Group
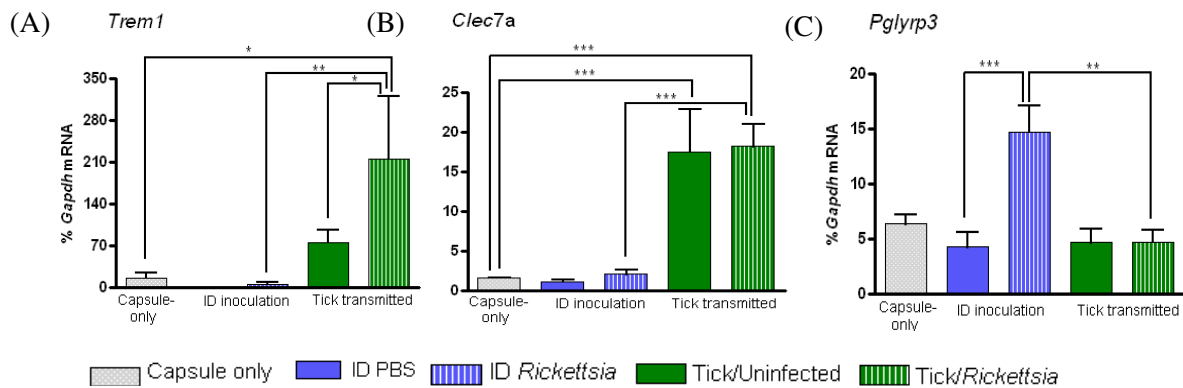

Figure. 22 mRNA expression of *Il-1b* (A) and *Casp-1* (B). Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *$p <0.05$; **$p <0.01$; and ***$p <0.001$

Bacterial recognition by TLR molecules and subsequent activation of MYD88 leads to

the increased production of the inflammatory cytokine IL-1β (Armant and Fenton, 2002).

Likewise, *IL-1β* mRNA was also upregulated in the tick transmitted group as compared to the ID

Rickettsia group (Fig. 22A).

Casp-1 is the regulatory molecule of IL-1β and it also helps in cell death by apoptosis and

host survival. mRNA expression of the *Casp*-1 gene was highly upregulated ($p<0.001$) in the

uninfected tick group compared to the *Rickettsia* infected tick group (Fig. 22B). This is

consistent with the increased expression of *Il-1β* in the infected tick group. Although the

production of mature IL-1β is dependent on Casp-1, regulation during tick infestation is not

identical.

4.4.2: Expression of *Hmox1/Ho-1* and *Ppbp* mRNA Was Upregulated in the Infected Tick Group Compared to Other Groups

Figure. 23 mRNA expression of *Hmox-1*. Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *$p <0.05$; **$p <0.01$; and ***$p <0.001$

Heme-oxygenase-1 (HMOX-1) is catalytically very active and responsible for degrading

heme to carbon monoxide, free iron and biliverdin. HMOX-1 is considered to be a very sensitive

indicator of oxidative stress in endothelial cells (Kushida et al., 2002; Yachie et al., 2003). In addition to this, a direct link exists between LPS, TLR4 and HMOX-1. Expression of HMOX-1 was significantly inhibited in TLR4 mutant mice (Song et al., 2003). The data also shows a correlation between *Tlr4* and *Hmox-1* mRNA expression, as both are upregulated in the *Rickettsia*-infected tick group. However, unlike *Tlr4*, *Hmox-1* mRNA expression was upregulated in the *Rickettsia*-infected tick group and was not upregulated in the uninfected tick group (Fig. 23).
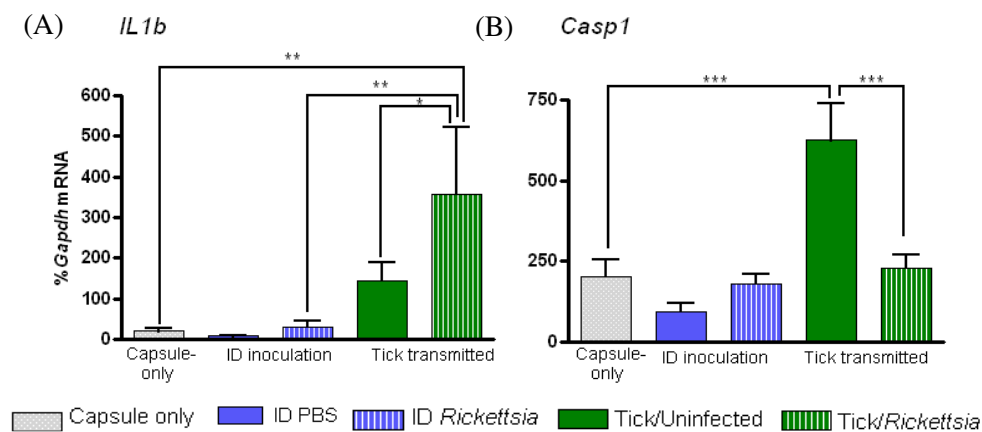

Figure. 24 mRNA expression of *Ppbp*. Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *p <0.05; **p <0.01; and ***p <0.001

Platelet basic protein (PBP) is a granular protein formed from platelets, which is derived from pro-platelet basic protein (PPBP). PBP in turn gives rise to several other derivatives that serve as anti-microbial peptides involved in bacterial killing. As shown in Fig.24, mRNA expression of *Ppbp* was highly upregulated in the infected tick group compared to the uninfected, as well as the ID inoculated *Rickettsia* group. However, *Rickettsia* were unable to induce the secretion of *Ppbp* mRNA when injected intradermally. As such, is possible that the induction of *Ppbp* is suppressed when *Rickettsia* is injected intradermally but that *Rickettsia* and

tick saliva together present multiple stimulators (i.e. TLR stimulatory effectors) that results in

increased PPBP production.

4.4.3: Expression of *Il-6* mRNA Was Highly Upregulated in Response to Uninfected Tick Infestation Compared to Infected Tick Infestation and Intradermal Rickettsial Infection

IL-6 acts as a pleotropic cytokine and regulates various functions, including

inflammation, acute-phase response, and differentiation of B-cells into antibody-producing

plasma cells. Although IL-6 can serve as both a pro- and anti-inflammatory molecule, it has been

shown that during intracellular infection by *Listeria monocytogenes* and *Mycobacterium*

tuberculosis, IL-6 plays a protective role (Dube et al., 2004; Hume et al., 2006). *Il-6* mRNA was

upregulated in the uninfected tick group compared to the capsule-only group, but was decreased

in the *Rickettsia*-infected tick group (Fig. 25). Thus, it is possible that *Rickettsia* infection
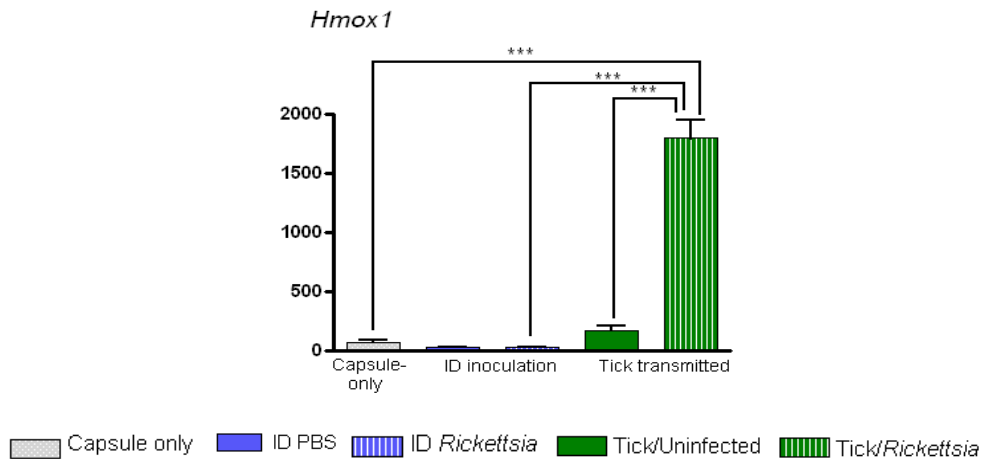
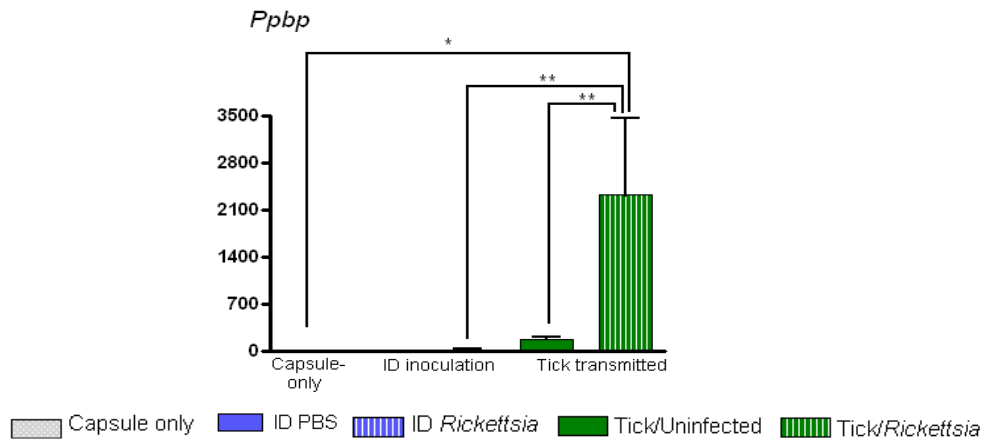suppresses *Il-6* mRNA expression induced by tick infestation.

Figure. 25 mRNA expression of *Il6*. Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *$p <0.05$; **$p <0.01$; and ***$p <0.001$

4.5: Expression of the Non-PRR Receptor *Ifngr1* mRNA Was Unaltered in All the Groups

IFNGR1 is expressed by NK cells and Th1 cells. Increased *Ifngr1*mRNA in the skin

could suggest an increase in the presence of NK cells or *Ifngr1* mRNA at the site of tick bite or

Rickettsia infection. SuperArray analysis indicated that *Ifngr1* mRNA was expressed at a greater

level in the tick-transmitted *Rickettsia* group than in the ID inoculated *Rickettsia* group.

However, real time PCR analysis did not demonstrate significant change in *Ifngr1* mRNA

expression in any of the groups (Fig. 26).
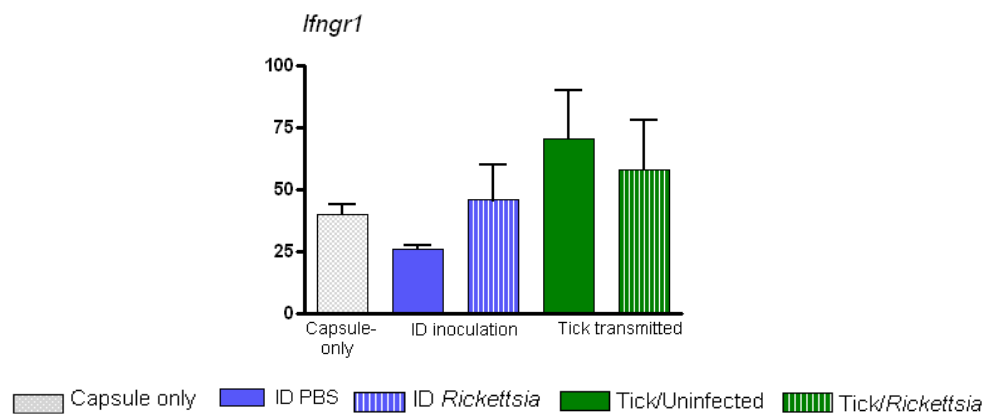
Figure. 26 mRNA expression of *Ifngr1*. Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *p <0.05; **p <0.01; and ***p <0.001

4.6: Role of Langerhans Cells as APCs During Rickettsial Infection and Tick Bite

4.6.1 Expression of *Langerin/Cd207* mRNA Was Upregulated in the Uninfected Tick Group

Langerin is considered to be the Langerhans cell marker and is only present on LCs.

Apart from being a marker, it was shown that langerin can act as an endocytic receptor and

potent inducer of Birbeck granules. Langerin binds to mannose-containing antigens from

pathogens and delivers them to the Birbeck granules in LCs (Mizumoto and Takashima, 2004).

The expression of *Langerin*/*Cd207* mRNA was upregulated in the uninfected tick group compared to both infected tick and needle inoculated *Rickettsia* group (Fig. 27). This could indicate an increased presence of LCs at infected tick bite site or an increase in *Cd207* mRNA by LCs at the site of infestation. Interestingly, *Rickettsia* infection did not enhance the tick-induced upregulation of *Cd207*mRNA, but instead suppressed *Cd207* mRNA expression.
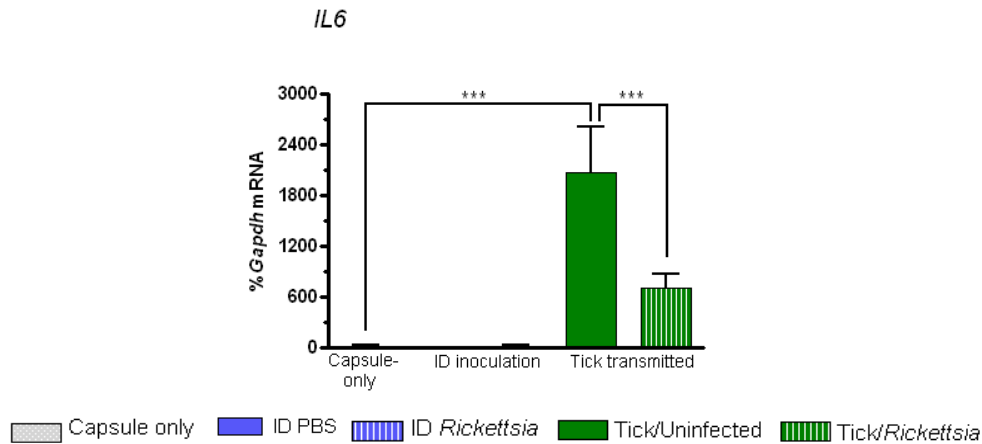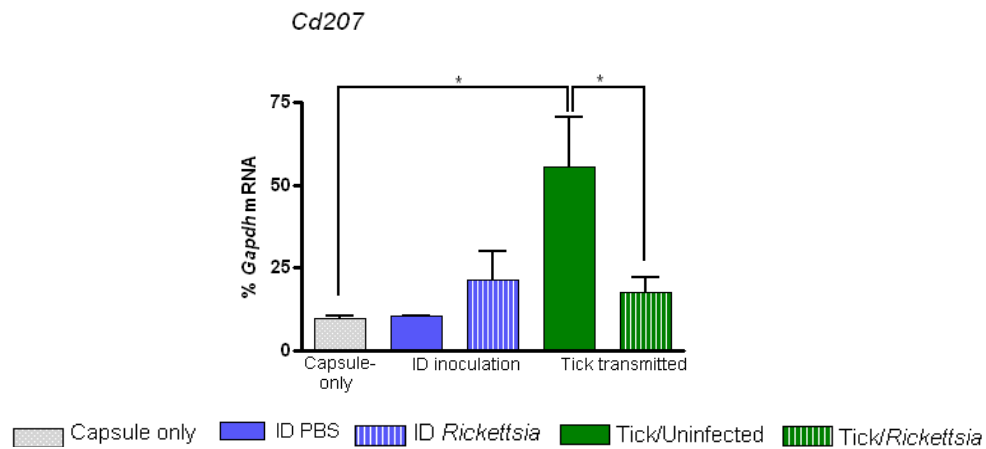

Figure. 27 mRNA expression of *Cd207*. Experiment was performed as previously described in Fig: 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *p <0.05; **p <0.01; and ***p <0.001

4.6.2: mRNA Expression of the Co-stimulatory Molecules *Cd80* and *Cd86* Present on APCs Were Upregulated in the Tick Infested Group Compared to the Needle-inoculated *Rickettsia* Group

CD80 and CD86 are the co-stimulatory molecules present on LCs and various other APCs in skin. Without these co-stimulatory molecules, LCs are unable to activate T cells and instead induce a state of anergy (reviewed by Daniel, L. Muller, 2000). *In vitro* studies have shown that while LPS alone can up-regulate the expression of CD80 and CD86, the addition of tick saliva can suppress the response of these molecules (Cavassani et al., 2005).

In the current study, tick infestation induced an up-regulation of *Cd80* and *Cd86* mRNA

compared to the needle-inoculated *Rickettsia* group (Fig. 28). *Cd80* mRNA was upregulated in

skin from *Rickettsia*-infected tick infestation, while *Cd86* mRNA was not. This suggests that

during tick infestation and rickettsial infection, both molecules are differentially regulated on the

APCs, and that in general there is an up-regulation of these molecules in presence of tick

infestation.
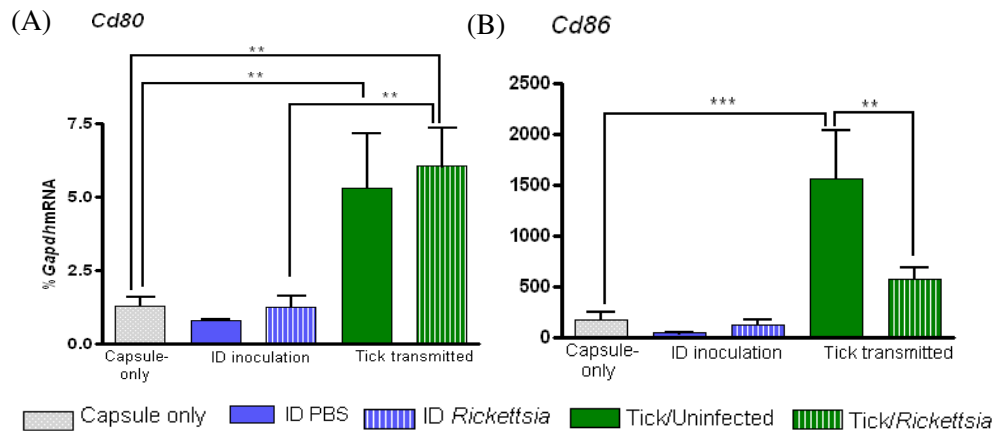
Figure. 28 mRNA expressions of *Cd80* (A) and (B) *Cd86*. Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *p <0.05; **p <0.01; and ***p <0.001

4.6.3: mRNA Expression of the Chemokine Receptor *Ccr1* and *Ccr5* Present on LCs Was Also Upregulated in the Tick Infested Group Compared to the Needle-inoculated *Rickettsia* Group

LCs are the major APCs in skin, resides in the epidermis that respond to different

chemokines during inflammation. These cells then migrate from the bloodstream to the site of

inflammation. Immature and mature LCs express different chemokine receptors on their surface

during their recruitment in skin and later during migration from the skin to draining lymph node.

CCR1 and CCR5 are expressed during their migration from the blood stream to the skin.
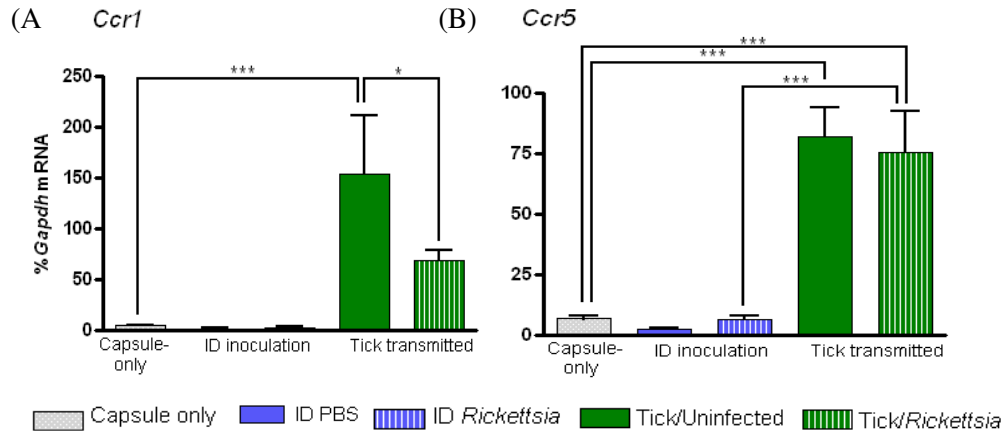
Figure. 29 mRNA expressions of chemokine receptor *Ccr1* (A) and *Ccr5* (B). Experiment was performed as previously described in Fig. 4. Data are the means ±SEM, n=3-5 mice per group. Statistical analysis was done by one-way ANOVA using Newman-Keuls post test. *p <0.05; **p <0.01; and ***p <0.001

In this study, both *Ccr1* and *Ccr5* mRNA were upregulated in the tick infested group

compared to the needle- inoculated *Rickettsia* group (Fig. 29). Possibly tick infestation results in

the recruitment of LCs to the site of infection which results in higher levels of *Ccr1* and

*Ccr5*mRNA expression.

Chapter 5: Discussion

In this study, we have analyzed the modulation of innate immune response genes in skin following rickettsial infection through either intradermal inoculation or tick transmission. First, SuperArray Real-Time PCR was performed to identify the differentially expressed mRNA of the innate and adaptive immune genes following intradermal versus tick transmission of *Rickettsia*. Next, Real-Time qPCR was utilized to confirm these results and to identify whether uninfected tick or *Rickettsia*-infected tick infestation was responsible for differences in mRNA expression. mRNA of *Hmox1, Il6, Ppbp, Md2, Lbp, Tlr4 Trem1, Il-1β* and *Clec7a* genes were found to be upregulated in the infected tick group and not in tick infestation alone, whereas mRNA of *Casp1, Il6, Cd207, Ccr1* and *Cd86* genes were upregulated in the tick infested group.

While the general trend is towards an agreement between these the SuperArray and Real-Time confirmation results, not all the results were similar between the two techniques. The mRNA expression of *Ifngr1*, *Traf6, Mapk8* and *Tollip* were unchanged in the traditional Real-Time PCR, but their expression was downregulated in the ID inoculated *Rickettsia* group in the SuperArray data. The mRNA expression of *Hmox1, Pglyrp3, Ppbp, Lbp, Tlr4* and *Trem1* were similar in both SuperArray and traditional Real-Time PCR. Discrepancies arising between the SuperArray and the quantitative Real-Time PCR data might be due to the technical differences between the procedures, as experimental reagents, cDNA preparation protocols as well as the primers used for amplification were different in each case. In addition, the length of the RT reaction time was also different, which might have resulted in different cDNA amounts. The differences in primer concentrations between the two sets of primers and in the PCR reaction volume might influence the results too. Again, given the sensitive nature of both these

techniques, some other factors which may cause non-trivial differences are the differences in ionic strength of the buffers used and last, but not the least, human pipetting error. In the current study we found that tick feeding and *Rickettsia* both can modulate the host immune response in a variety of ways. Interestingly, in some cases, tick-transmitted *Rickettsia* exerted different immune response compared to the ID inoculation. These differences in expression induced via ID-inoculated and tick-transmitted *Rickettsia* might be due to the fact that *Rickettsia* might induce different immune response depending on the route of transmission, as has been shown with *Borrelia burgdorferi* (Roehrig et al., 1992). Differences in gene expression profile or surface protein expression of *Rickettsia* can induce different immune response during infection. Another possibility might be that when *Rickettsia* are transmitted via ticks, some of the rickettsial proteins or components are masked by the tick salivary gland proteins, allowing the bacteria to exploit the immunomodulatory properties of the tick saliva.

5.1: Langerhans Cells and PRR Response to ID Inoculated and Tick Transmitted *Rickettsia*

Langerhans cells are a type of dendritic cell in the skin epidermis that play a critical role as antigen presenting cells (Romani et al, 2003). LCs express many cell-surface receptors including TLR2, TLR1, TLR2, TLR3, TLR6 (Flacher et al., 2006), Fcγ, Fcε and langerin/CD207, which is C-type II lectin receptor and acts as a marker for LCs (Valladeau et al., 2000). In several studies it was shown that LCs can trap tick salivary gland antigen in guinea pigs and present those antigens to the draining lymph nodes (Allen et al., 1979; Nithiuthai and Allen, 1985). Contrastingly, it was shown that tick saliva can impair the maturation, migration and antigen presenting capacity of skin DCs (Skallova et al., 2008). Data suggested that LCs may

be recruited to the tick bite site, because tick infested skin tissues showed an increased mRNA expression of the LC marker *Cd207* and activation markers *Cd80, Cd86*, as well as the chemokine receptors *Ccr1* and *Ccr5*. It might also be possible that LCs were activated in response to the tick bite and activated cells induced mRNA expression of the above genes. To explain whether LCs were able to migrate to the draining lymph node and present the antigens to T cells, further studies are needed to identify the expression of maturation a marker *i.e* CCR7, on LCs and the LC response in the draining lymph node.

During inflammation and pathogen infection, immune cells including macrophages, neutrophils or basophils are recruited to the site of infection or inflammation to mount an efficient immune response. Different cell populations express different PRRs on their surface, for example, dermal endothelial cells express TLR2, TLR4 and TLR9 (Faure et al., 2000), whereas LCs do not express TLR4 or TLR9 and only monocytes and *in vitro* generated DCs express TLR4 (reviewed by (Iwasaki and Medzhitov, 2004). The mRNA expression of *Tlr4* was high in the tick infested group, suggesting that cells expressing TLR4 were also recruited to the site of the tick bite other than LCs.

The expression of the PRRs, including *Clec7a* and *Trem-1*, was high in the tick infested group compared to both the capsule only control and ID inoculated *Rickettsia* groups, which indicates that, apart from LCs, many other immune cells are also recruited at the site of tick bite including natural killer cells, macrophages and neutrophils which express the PRRs CLEC7A and TREM-1 on their cell surface. As TREM-1 and TLRs are expressed on neutrophils and monocytes, it is expected that *Rickettsia*-infected tick infestation will cause an increase in the

expression of *Trem-1* and *Tlr* mRNA expression because both tick bites and *Rickettsia* infection cause an influx of neutrophils and monocytes (Sporn and Marder, 1996); reviewed by (Wikel, 1999), which in turn might increase the mRNA expression of the above genes.

The response of *Tlr9* and *Pglyrp3* mRNA was different than the response from the other PRRs. *Tlr9* mRNA was downregulated in the tick infested tissues compared to the capsule-only control. It was shown that tick saliva can inhibit the maturation of DCs upon TLR9 ligation (Skallova et al., 2008), suggesting that the inhibition of DC maturation during tick infestation is due to the decreased expression of *Tlr9* mRNA or to the unresponsiveness of TLR9 on DCs. Histological studies needed to evaluate the TLR9 response on DCs before concluding that ticks inhibit DC activity. Although *Tlr9* mRNA expression was not influenced in the ID-inoculated *Rickettsia* group, its expression was higher in the *Rickettsia*-infected tick group. On the other hand, *Pglyrp3* mRNA was highly upregulated in the ID inoculated *Rickettsia* group compared to the PBS control group. The *Pglyrp3* mRNA expression was unaltered in the *Rickettsia*-infected tick group. This suggests that *Rickettsia* may influence the mRNA expressions of these two genes.

During bacterial infection several other molecules help the PRRs in recognizing bacterial components. The molecule responsible for binding bacterial LPS is LPS-binding protein (LBP) (Tobias et al., 1986). Even low amounts of LBP can cause efficient binding to LPS and help to promote host's inflammatory response through activation of TLR4 (Kitchens and Thompson, 2005). In our data, *Lbp* as well as *Md2* mRNA was upregulated in the tick infested group, especially in the *Rickettsia*-infected tick group compared to the capsule-only control. When the

ID inoculated *Rickettsia* group and tick-transmitted *Rickettsia* groups are compared, there was a significant upregulation in the *Rickettsia*-infected tick group. This difference could be due to different cell types in the skin tissues or a different level of cell activation.

Similar to the *Lbp* and *Md2* mRNA expression, *Myd88* mRNA expression in the ID inoculated *Rickettsia* group was almost similar to its PBS control groups and its expression was higher in the tick infested group. The increased mRNA expression of *Myd88* in the tick infested group might be due to the recruitment of different antigen presenting cells expressing TLRs and MyD88 during tick infestation which subsequently increased the MyD88 mRNA expression. In contrast to the high mRNA expression of *Tlr2*, *Tlr4* and *Myd88* during tick infestation, mRNA expression of *Tollip, Trfa6* and *Mapk8* were unaltered during both tick infestation and *Rickettsia* infection. The unaltered mRNA expression of the adaptor molecules, in spite of high PRR mRNA expression, might occur because the signal transduction genes are transcribed and translated at a high level, with a low turnover rate, and are not induced by the TLR mediated cell activation. It might also be possible that these genes are controlled by different signal cascades or are regulated differently on different cell types that might produce different amount of signal transduction genes mRNA. Further investigation is needed to see the protein expression level of the adaptor molecules to confirm their activation or inactivation.

5.3: Tick-transmitted *Rickettsia* Infection Influenced the Expression of *Ifngr1*, *Hmox-1*, *Il-1β* and *Ppbp* mRNA

The recognition of microbial components by various PRRs, including TLRs, TREM-1, PGLYRP3, CLEC7A, as well as cytokine stimulation of cells via IFNGR1 induces the activation of immune cells including macrophages, dendritic cells, NK cell and neutrophils. The activated

cells subsequently synthesize and secrete several cytokines, including IL-1, TNF-α, IL-6, IFN-γ, chemokines, including MCP-1, CCL1, CCL17, CCL5, and several other components including Casp-1, HMOX-1, PPBP by these cells in an effort to fight the invading microbes and ultimately aid in efficient microbial killing.

Although the SuperArray data showed an increase in *Ifngr1* mRNA in the tick-transmitted *Rickettsia* group compared to the ID-inoculated *Rickettsia* group, the real-time PCR showed an unaltered expression of *Ifngr1* mRNA expression. This might be due to the fact that as IFN-γ and TNF-α induced macrophage play a major role in *Rickettsia* killing (Feng and Walker, 2000). During tick-transmitted *Rickettsia* infection the increased amount of inflammatory cytokines TNF-α and IL-1 may enhance macrophage accumulation at the infection site which in turn increases the *Ifngr1* mRNA expression or the cytokines actually activated the cells to express more IFNGR1 receptors. The activated IFNGR1 or the increased number of IFNGR1 receptors on macrophages can bind to IFN-γ and might induce activation of macrophages.

Rickettsial infection can lead to an increased expression of both HMOX-1 and expression IL-6, as it was shown for HMOX-1 production in human endothelial cells infected with *R. rickettsii* (Rydkina et al., 2002) and IL-6 production in *Rickettsia conorii* infected human umbilical vein endothelial cells (HUVEC) showed increased amount of IL-6 production (Kaplanski et al., 1995). On the other hand, tick salivary gland extract can suppress the production of IL-6 in LPS activated cells (Fuchsberger et al., 1995). Interestingly, our data showed no significant change of *Il-6* or *Hmox-1* expression between the ID inoculated *Rickettsia*

and PBS control groups. *Il-6* mRNA was highly upregulated in the uninfected tick group compared to the *Rickettsia*-infected tick group and *Hmox-1* mRNA was highly upregulated in the *Rickettsia*-infected tick group. This indicates that *Rickettsia* is transmitted via tick, might be able to induce the upregulation of *Hmox-1* mRNA and downregulation of *Il-6* mRNA to further suppress the immune reactions induced by IL-6.

Similar to *Hmox-1* mRNA expression, *Il-1β* and *Ppbp* mRNA expression was also high in the tick-transmitted *Rickettsia* group compared to the ID-inoculated *Rickettsia* group and the uninfected tick group. Peripheral blood leukocytes treated with both LPS and salivary gland extract of *Rhipicephalus appendiculatus* salivary gland extract had reduced expression of IL-1β compared to only LPS treated cells (Fuchsberger et al., 1995). Enhanced expression of *Ppbp* and *Il-1β* mRNA in the tick-transmitted *Rickettsia* group might be due to the recruitment of different cell populations during uninfected tick and infected tick infestation, which in turn produces different levels of mRNA.

Casp-1 is responsible for cleaving pro-IL-1β and pro-IL-18 to mature IL-1β and IL-18 as well as inducing apoptosis-mediated death (Miggin et al., 2007). Our result showed an up-regulation of *Casp-1* in the uninfected tick group compared to both infected tick and the ID inoculated *Rickettsia* group. This upregulation od *Casp-1* in the uninfected tick is in contrast with a lack of *Il-1* mRNA upregulation. Instead *Il-1* is upregulated at a high level in the infected tick group. This was surprising as one would expect both IL-1β and Casp-1 to be upregulated on the same cell type and by the same promoter to make efficient production of active IL-1β.

In conclusion, the skin immune response during rickettsial infection, both via intradermal inoculation and tick transmission, induced the upregulation of a wide array of immune cells and molecules. Although we have demonstrated that tick infestation was able to upregulate the *Tlr2* and *Tlr4* mRNA expressions, we were unable to conclude whether it caused the activation of the TLRs and the down-stream adaptor molecules to further activate NF-κB. While some cells and molecules were differentially regulated during the tick infestation and rickettsial infection, a consistent direction of modulation was observed for some genes when *Rickettsia* was transmitted via its natural route, i.e. tick saliva. This modulation could favor either rickettsial replication inside the infected cells or dissemination of *Rickettsia* into the blood to establish systemic infection. Although it is known that tick saliva can suppress a variety of immune responses including the modulation of complement activation, natural-killer cell function, antibody production, inhibition of T-lymphocyte responsiveness, and dendritic cell activation and maturation (reviewed in (Wikel, 1996), it is unclear at this point if *Rickettsia* is unilaterally taking advantage of the tick immune modulatory capabilities or if *Rickettsia* is also benefiting the tick in some way. Overall, our data suggests that the innate immune response to *Rickettsia* infection is substantially different in the skin when *Rickettsia* is transmitted via tick versus ID inoculation.

As *R. amblyommii* is non-pathogenic to the human host, it is also possible that during intradermal injection of *R. amblyommii*, mice were able to clear the infection by 5-7 days, which was reflected in the low mRNA expression of several genes. Another possibility is that during rickettsial transmission via tick, tick delivered a much higher amount of *Rickettsia* to the mice compared to needle inoculation which is reflected in higher mRNA levels of the immune

response genes. To answer this question, further study should be done to standardize the rickettsial dose delivered via needle which is comparable to natural infection via tick. Infection with pathogenic *Rickettsia*, for example *R. rickettsii* or *R. parkerii* might help to understand the differences or similarities in immune response generated against the pathogenic and non-pathogenic *Rickettsia*. Because gene expression from a specific cell population cannot be identified by the qPCR analysis of whole tissue, future studies will be needed to understand the detailed mechanism of how several PRRs and immune cells are modulated by *Rickettsia* when it is transmitted by ticks.

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