# Host and Pathogen Sensory Systems as Targets for Therapeutic Intervention

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By

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**Abstract**

A new paradigm for the treatment of infectious disease is through the modulation of innate immune responses. In this capacity, host defense peptides (HDPs) and synthetic Toll-like receptor 9 (TLR9) ligands have the greatest demonstrated potentials. The work presented here considers mechanisms for the improvement of these treatments through optimization, or in the case of HDPs the minimization, of the interactions of these ligands with sensory receptors.

Toll-like Receptor 9 activates the innate immune system in response to microbial DNA or immune-modulating oligodeoxynucleotides. While cell stimulation experiments demonstrate the preferential activating ability of CpG-containing nucleic acids, direct binding investigations have reached contradictory conclusions regarding the sequence-specificity of TLR9 ligand binding. To address this discrepancy the characterization of human TLR9 ligand binding properties is reported. TLR9 has a high degree of ligand specificity in being able to discriminate not only CpG dinucleotides, but also higher order six nucleotide motifs that mediate species-specific activation. However, TLR9 ligand binding is also functionally influenced by nucleic acids in a sequence-independent manner both *in vitro* and in cell proliferation experiments. A model is proposed in which TLR9 activation is mediated specifically by CpG-containing ligands while sensitivity of the receptor is modulated by the absolute concentration of nucleic acids in a sequence-independent fashion.

Host defense peptides are among the leading candidates to combat antibiotic resistant bacterial strains. Recently, HDPs have been demonstrated to function as ligands for the bacterial sensory kinase PhoQ resulting in the induction of virulence and

adaptive responses. Thus, concerns have been raised regarding therapeutic applications of HDPs. Here a methodology is described that permits discrimination and quantification of the distinct, but related, peptide behaviors of direct antimicrobial activity and PhoQ ligand potential. Utilizing peptide derivatives of the model HDP Bac2A it is demonstrated that antimicrobial efficiency is significantly, and inversely, related to PhoQ ligand efficacy. This provides a rational basis for HDP selection with greater therapeutic potential and minimized potential for initiation of bacterial resistance.

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## List of Abbreviations

| | |
|---|---|
| AEMSA | Agarose electrophoretic mobility shift assay |
| APC | Antigen presenting cell |
| ATP | Adenosine triphosphate |
| BDR | Bacterial defensive response |
| BMAP | Bovine Myeloid Antimicrobial Peptide |
| BSA | Bovine serum albumin |
| C | Cytosine |
| CD | Circular dichroism |
| CF | Cystic fibrosis |
| CpG | Cytosine-phosphate-guanine |
| CMV | pFLAG-CMV-2 |
| dAMA | Direct antimicrobial activity |
| DNA | Deoxyribonucleic acid |
| ds | Double-stranded |
| DOTAP | 1,2-dioleoyl-3-trimethylammonium propane |
| DTT | Dithiothreitol |
| EMSA | Electrophoretic mobility shift assay |
| EDTA | Ethylenediaminetetraacetic acid |
| EtBr | Ethidium Bromide |
| G | Guanine |
| GpC | Guanine-phosphate-cytosine |
| HDP | Host defense peptides |

| | |
|---|---|
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL-1 | Interleukin-1 |
| IL-1R | Interleukin-1 receptor |
| IRAK | IL-1 receptor-associated kinase |
| IRF | Interferon regulatory factor |
| JNK | c-Jun N-terminal kinase |
| LB | Luria broth |
| LBP | LPS-binding protein |
| LPS | Lipopolysaccharide |
| LRR | Leucine-rich repeat |
| LTA | Lipoteichoic acid |
| MHC | Measurement of hemolytic activity |
| MIC | Minimum inhibitory concentration |
| MyD88 | Myeloid differentiation primary response protein 88 |
| NFκB | Nuclear Factor kappaB |
| NK | Natural killer |
| ODN | Oligodeoxynucleotide |
| PAGE | Polyacrylamide gel electrophoresis |
| PAMP | Pathogen-associated molecular pattern |
| PBMC | Peripheral blood mononuclear cells |
| PD | Phosphodiester |
| pDC | Plasmacytoid dendritic cells |
| PE | Peptide efficiency |

| | |
|---|---|
| pH | Negative logarithm of the hydrogen ion concentration |
| PhoPQ | PhoP-PhoQ two-component system |
| pKa | Negative logarithm of the dissociation constant of an acid |
| poly(I:C) | Polyriboinosinic-polyribocytidylic acid |
| PRR | Pathogen Recognition Receptor |
| PTO | Phosphothioate |
| PTM | Post-translational modification |
| RI | Retro-inverso |
| RNA | Ribonucleic acid |
| SDS | Sodium dodecyl sulfate |
| SLE | Systemic Lupus Erythematosus |
| SPR | Surface Plasmon Resonance |
| ss | Single-stranded |
| TBST | Tris-buffered saline with Tween |
| TCS | Two-component system |
| TFE | Trifluoroethanol |
| TIR | Toll/IL-1R domain |
| TLR | Toll-like receptor |
| TLR9(LBD) | Toll-like receptor 9 ligand-binding domain |
| TNF-α | Tumor Necrosis Factor-alpha |

# 1.0 Introduction

For millions of years, hosts and pathogens have been in constant contact with one another. As a result, both organisms have undergone a dynamic co-evolutionary process resulting from significant selective pressures exerted by one upon the other. These pressures have resulted in the development of offensive and defensive strategies centered on mutual inhibition, evasion and adaptive strategies (Peschel 2006).

Central to the offensive and defensive strategies of these organisms are sensory systems that constantly monitor both the internal and external cellular environments. Host organisms rely on the innate immune system, and in particular the Toll-like receptor system, for early detection of microbial invasion and for the initiation of an appropriate response to limit pathogen colonization. Microbial organisms rely on similar sensory systems for the initiation of adaptive responses – the two-component sensory system. Although much less complex than the Toll-like receptor system of the host, two-component sensory systems increase the ability of the microbe to resist the antimicrobial arsenal of the innate immune system.

## 1.1 The Innate Immune System

The innate immune system is a universal and ancient form of host defense against infectious organisms. The central role of this system is the recognition of microbial invasion and the initiation of responses that limit the spread of pathogenic

microorganisms throughout host tissues (Janeway 2002). The innate immune system utilizes a limited number of germline-encoded receptors to distinguish self from non-self biomolecules through conserved microbial patterns. As a testament to the success of the innate immune system, invertebrates, comprising more species in nature than vertebrates, rely exclusively on this system to mount host defensive responses. In contrast, vertebrates also utilize the adaptive immune system to combat pathogen invasion. The adaptive immune system functions to generate immunological memory thus providing adaptive fitness. This response relies on the generation of antigen receptors (the T- and B-cell receptors, TCR and BCR, respectively) whose broad diversity is based on random gene rearrangements. However, the activation of these antigen receptors results in a delayed response due to the clonal distribution of the antigen receptors. As this delayed response takes approximately 4-7 days it is too slow for rapidly-replicating organisms such as microbes (Janeway 2002). Thus, the innate immune system is indispensable as the primary defensive response against pathogenic microbes.

During innate immune system activation, four fundamental activities are undertaken: 1) rapid detection of infectious agents, 2) categorization of the cellular location (intracellular or extracellular) of the pathogen, 3) elimination or containment of the pathogen, and 4) induction of an appropriate adaptive immune response for pathogen eradication and prevention of recurrent infection (Krieg 2006). At the center of these activities are the pathogen recognition receptors (PRRs).

The pathogen recognition receptors are non clonal, germline-encoded receptors that monitor the intracellular and extracellular environments for pathogen invasion. Although limited in number, the PRRs have evolved the ability to recognize highly

diverse microbial organisms through recognition of invariable molecular structures. These structures include molecules such as outer cell membrane components [lipoteichoic acid (LTA), lipopolysaccharide (LPS)], nucleic acids (DNA and RNA) and proteins (flagellin) (Janeway 2002). As these conserved structures cannot be easily altered by microorganisms they have remained fairly invariable throughout microbial evolution but are absent or highly suppressed within the host. Although not identical across microbial species, the microbial patterns recognized by the PRRs are always found within the context of a conserved molecular structure. The conserved molecular patterns recognized by PRRs allow for several strain- and species-specific variations (Janeway 1989). As an example, Gram-negative bacterial species exhibit significant variance amongst LPS molecules, particularly within the O-antigen portion. The PRR Toll-like receptor 4 has, however, evolved to recognize a conserved molecular pattern within LPS, the lipid-A portion. Accordingly, these microbial biomolecules have been termed microbe- or pathogen-associated molecular patterns, MAMPs or PAMPs, and describe the conserved microbial targets of the innate immune system (Watson 2006).

As targets of the innate immune system, PAMPs have three conserved features (Medzhitov 2001). Firstly, PAMPs are microbial-specific biomolecules that are absent or suppressed within the host cell. This broad definition is not completely unambiguous as activation of innate immune responses may in some instances be triggered to self-biomolecules. For example, hypomethylated cytosine-phosphate-guanine (CpG) motifs, although a PAMP for Toll-like receptor 9, are found within host organisms, albeit in reduced frequency in comparison to microbial DNA. Secondly, PAMPs are absolutely required for microbial survival; hence their invariance across microbial classes. Lastly, as demonstrated by the lipid-A portion of LPS, PAMPs are invariant between

microorganisms of a given class. This recognition of invariant patterns has reduced the total number of PRRs required to mount an innate immune response and is typified by the Toll-like receptor (TLR) family.

## 1.2 Drosophila Toll and TLR Identification

The discovery of Drosophila Toll, the first member of the Toll family, was not predicated on it being a PRR involved in innate immune activation. Rather, Toll was one of twelve maternal effector genes found to control dorsoventral axis formation in fruit fly embryos (Belvin 1996, Hashimoto 1988). The Toll system was found to involve a Toll ligand (Spätzle), an adapter protein (Tube), and a protein kinase (Pelle). An NF-kB family transcription factor (Dorsal) and the Dorsal inhibitor (Cactus) were also found to be involved in the Toll system.

The role of Toll in the innate immune response of Drosophila was suggested by the homology between Toll and the human Interleukin-1 (IL-1) receptor (IL-1R). The intracellular domains of these receptors were found to be homologous and suggested conserved functions (Janeway 2002). In support of this, activation of either receptor resulted in the induction of Nuclear Factor kappaB (NF-kB) activation, with a concensus NF-kB binding site found in the promoter regions of Drosophila antimicrobial peptides (Engstrom 1993). It was also found that signaling through Toll and IL-1R proceeded through homologous protein kinases, Pelle (in the case of Toll) and IL-1 receptor-associated kinase (IRAK) in the case of IL-1R (Belvin 1996, Anderson 2000).

As activation of Toll leads to induction of antimicrobial peptide transcription, and antimicrobial peptides are rapidly induced in response to infection, a connection

between Toll and the induction of Drosophila immune responses was suggested. Furthermore, Toll mutants of Drosophila rapidly succumbed to fungal infection and fruit flies with loss-of-function mutations to *spatzle*, *tube* or *pelle* were also highly susceptible to fungal infections (Lemaitre 1996). Thus, the Toll pathway controls both dorsoventral patterning within embryos and the antifungal immune defense in adult fruit flies (Medzhitov 2001). The recognition of Toll's involvement in Drosophila innate immune responses led to the identification of a family of Toll homologues in vertebrates.

### 1.3 The Toll-like Receptors

Following the identification of Toll and its central role in the immune response of Drosophila, homology searches in vertebrates uncovered the first characterized Toll homologue, Toll-like receptor (TLR) 4. The role of TLR4 in the vertebrate innate immune system was suggested following demonstration that a constitutively active form of TLR4 was able to induce inflammatory cytokine expression (Medzhitov 1997a). Subsequent studies confirmed the role of TLR4 in the induction of innate immune responses with lipopolysaccharide (LPS) identified as the ligand for TLR4 (Poltorak 1998, Qureshi 1999, Hoshino 1999). This has culminated in the identification of 13 TLR family members in mammals and 10 members in the human family.

The mammalian TLRs are activated by unique ligands that share diversity in structure and origin. Thus, three common themes have emerged regarding the relationship between the receptors and their ligands: 1) the majority of TLR ligands are PAMPs (in contrast endogenous nucleic acids, heat-shock proteins, and extracellular

matrix breakdown products have been regarded as self-TLR ligands), 2) TLRs can recognize structurally unrelated ligands, and 3) some TLRs require accessory proteins for ligand recognition (Medzhitov 2001). The TLR family also displays diversity between species in regards to expression and function: TLRs 1 through 9 are conserved in both mice and humans, yet TLR10 is present only in humans and TLR11 (also known as TLR12) and TLR13 have only been characterized in mice (Akira 2004).

### 1.3.1 Members of the Toll-like Receptor Family

**TLR1, TLR2, and TLR6:**

LPS was originally considered to be the ligand for TLR2; however, subsequent studies challenged this hypothesis. Wetzler demonstrated that contaminating bacterial lipoprotein in the LPS preparations was responsible for TLR2 activation (Wetzler, 2003). More recently, TLR2 ligands have been broadened to include Gram-positive peptidoglycan (Takeuchi 1999), mycobacterial cell-wall lipoarabinomannan (Underhill 1999a), *Trypanosoma cruzi* glycosylphophatidylinositol (Campos 2001), *Staphylococcus epidermidis* modulin (Hajjar 2001), and yeast cell walls (Underhill 1999b).

That TLR2 recognizes such a broad-range of ligands is a function of heterodimerization events with TLR1 or TLR6; associations which dictate ligand binding specificities (Ozinsky 2000, Takeuchi 2001). For example, the TLR2/TLR1 heterodimer recognizes a variety of lipoproteins whereas the TLR2/TLR6 heterodimer is involved in recognition of diacylated lipoproteins (Takeuchi 2002, Wetzler 2003, Takeuchi 2001). In addition, it has been speculated that TLR2 possibly functions as a homodimer, or heterodimer with an as of yet unidentified TLR, in the recognition and

binding of lipoteichoic acid (LTA), liparabinomannan, porins, and atypical LPS molecules (Massari 2002, Wetzler 2003). The expression of TLR2 has been found to be regulated and restricted to antigen-presenting cells and endothelial cells (Muzio 2000) whereas the expression of TLR1 and TLR6 are constitutively expressed on a variety of cell types (Medzhitov 2001).

**TLR4**

TLR4, the first characterized Toll-like receptor, remains the most well studied of the TLR family largely due to its role in sepsis. Trace amounts of LPS, the TLR4 ligand, activate the innate immune system and produce numerous proinflammatory mediators, such as Tumor Necrosis Factor-alpha (TNF-α), IL-1, and IL-6 (Akira 2004). During sepsis, TLR4 hyperactivity results in excessive proinflammatory responses ultimately leading to cell and tissue injury (Dauphinee 2006). Indeed, sepsis is the leading cause of mortality in critically ill patients associated with approximately 200,000 deaths per year in the United States (Angus 2001).

TLR4 is expressed in a variety of cell types, predominantly those of the immune system (Poltorak 1998, Qureshi 1999, Hoshino 1999). The recognition event between LPS and TLR4 requires several accessory proteins and the formation of a TLR4 complex with several membrane-linked and soluble molecules, including CD14 and MD-2 (West 2006). LPS-binding protein (LBP), a serum protein, first binds LPS and transfers the LPS monomer to CD14, a high-affinity LPS leucine-rich repeat (LRR) receptor. CD14 in turn is thought to transfer LPS to the TLR4 complex at the cell membrane (Ulevitch 1993, Wright 1990).

Although LPS is the most well characterized ligand for TLR4, TLR4 is also suggested in the recognition of LTA and heat-shock protein 60 (HSP60). The recognition of HSP60, although not well understood, has been suggested to represent a necessary inflammatory response due to necrotic cells (Medzhitov 2001) and has been speculated to have a role in tissue re-modeling and wound healing (Li 2001)

**TLR5**

TLR5 is involved in the recognition of flagellin, the Gram-negative bacterial flagella protein (Hayashi 2001). The recognition of ligand by TLR5 relies on a highly conserved, central core structure of flagellin essential for protofilament assembly and bacterial motility (Smith 2003). The TLR5 recognition site is presumed to be highly conserved amino- and carboxy-termini sequences within the central core of flagellin (Samatey 2001). A binding site for flagellin on the TLR5 ectodomain is speculated to be present between residues 386-407 as mutants lacking this domain are unable to interact with flagellin *in vitro* (Mizel 2003). As flagellin is proteinaceous in nature and does not contain atypical features, such as post-translational modifications, it may share similarities to host proteins and thus potentially remove the self-tolerance found within the innate immune system.

As TLR5 expression is polarized to the basolateral side of the intestinal epithelium, this might provide a unique feature that allows for the discrimination of commensal and pathogenic bacterium since the epithelial barrier is crossed by pathogenic bacteria but not commensal flora (Medzhitov 2001).

**TLR3, TLR7, TLR8, and TLR9**

TLR3, -7, -8, and -9 represent a subset of TLRs that interact with both RNA and DNA nucleic acids. These PAMPs are of particular importance because of their close resemblance to host molecules. In comparison to the cell surface-localized TLRs already described, this subset of TLRs is localized to intracellular organelles. This cytoplasmic shielding of the nucleic acid-activated TLR subfamily has been suggested as a potential safety mechanism to ensure differentiation of self- and non-self nucleic acids (Akira 2006).

**TLR3**

TLR3 expression confers responsiveness to purified dsRNA and polyriboinosinic polyribocytidylic acid [poly(I:C)] in cultured cells and TLR3-deficient mice display impaired responses to these ligands (Akira 2004). Upon activation, TLR3 induces NF-kB and Interferon (IFN) regulatory factor 3 (IRF3), ultimately leading to the production of antiviral molecules such as type I IFN (Alexopoulou 2001). As the PAMP for TLR3 is dsRNA, this receptor is involved in virus recognition during infectious cycles. dsRNA is a molecular pattern native to the vast majority of viruses and is generated during viral infection as a replication cycle intermediate (Akira 2006). TLR3 is largely expressed in cells that function as primary barriers to infection such as epithelial cells (including airway, uterine, corneal, vaginal, cervical, biliary, and intestinal) (Akira 2006).

Although the recognition of dsRNA by TLR3 is suggestive of a role in viral immunity, it has been demonstrated that TLR3 is not required for the initial induction of type I IFN during viral infection (Edelmann 2004). This suggests that other PRRs may be involved in dsRNA recognition.

**TLR7/8**

The homologous Toll-like receptors TLR7 and TLR8 recognize PAMPs in a species-specific manner. Mouse /human TLR7 and human TLR8 recognize guanosine- or uridine-rich single-stranded RNA (ssRNA) derived from RNA viruses (Diebold 2004, Heil 2003, Lund 2004). In addition, both receptors are also responsive to synthetic antiviral imidazoquinoline components and some guanine nucleotide analogs (Akira 2006). Mouse TLR8, although expressed, appears to be non-functional (Akira 2006).

As these receptors are responsive to ssRNA of both host and viral origin (Hemmi 2002, Heil 2004, Diebold 2004) it is likely that their endosomal localization acts as a safety mechanism by reducing the likelihood of such an interaction. Extracellular RNases also act as endogenous safeguards against such an event (Akira 2006). Additionally, it has been demonstrated that RNA of host origin is significantly less stimulatory that bacterial or viral RNA, suggestive of a host modification strategy to distinguish self-RNA from those of non-self origin (Kariko 2005).

**TLR9**

TLR9, the last of the endosomal TLRs, is unique amongst the TLRs as the only receptor that is responsive to both prokaryotic and viral DNA. This recognition is defined by unmethylated CpG dinucleotides within a particular base context (CpG-DNA). CpG-DNA induces powerful immune responses and TLR9-deficient mice are unresponsive to challenge with CpG-DNA (Hemmi 2000). That CpG-DNA is both highly modified by methylation and suppressed in frequency within the host genome reduces the potential of this receptor to be activated by endogenous DNA.

Cellular patterns of TLR9 expression are inconsistent amongst different species. In humans, TLR9 is expressed primarily in B cells and plasmacytoid dendritic cells (pDC) but has also been reported to be functionally expressed in activated neutrophils and pulmonary epithelial cells (Krieg 2006). Mouse TLR9, although expressed in pDC and B cells, is also expressed in monocytes and myeloid dendritic cells (Iwasaki 2004). As a consequence of this species-variance it is difficult to make predictions regarding TLR9-mediated responses based on extrapolation of results from different species (Krieg 2006).

In addition to bacterial DNA, TLR9 is also activated by viral DNA sequences that contain unmethylated CpG sequences. The genomes of DNA viruses, such as herpes simplex virus 1 and 2 (HSV-1 and HSV-2) and murine cytomegalovirus (MCMV), are rich in hypomethylated CpG motifs. The activation of TLR9 by viral CpG-DNA results in the induction of inflammatory cytokines and type I IFN secretion in a TLR9-dependent manner (Hochrein 2004, Tabeta 2004, Krug 2004, Lund 2003).

Although much is known about the ligands and signaling pathways of TLRs 1-9 and 11, the biological roles of TLRs 10, 12, and 13 remain unclear, as their expression patterns, ligands, and modes of signaling have yet to be defined (Akira 2004).

### 1.3.2 TLR-ligand Binding: The Leucine-Rich Repeat Domain

A conserved feature of the ligand-binding domains of Toll-like receptors is the presence of leucine-rich-repeat (LRR) motifs. TLR ectodomains are comprised of 19-25 tandem copies of an LRR motif, capped on either end by characteristic N- and C-terminal structures (Takahashi 1985). The individual LRRs are comprised of a 24-

amino acid consensus sequences XLXXLXLXXN (residues 1-10), conserved in all LRR subtypes, and XθXXθXXXXFXXLX (residues 11-24, where θ is a hydrophobic residue) (Spitzer 2002).

Many three-dimensional structures of proteins that contain LRR motifs have been solved and demonstrate that each LRR forms a loop structure. As a result, the juxtaposition of several LRRs results in an overall coiled or solenoid-like horseshoe structure (Bell 2003). Within individual LRRs, the first 10 residues forms a beta-strand and the remaining residues form an alpha-helix (Bell 2003). As a result, a large concave surface is formed by a repeating array of beta-sheets, in concurrence with the overall solenoidal shape of the LRR domain, and a convex surface formed by alpha-helices.

Based on structural investigations of proteins containing LRR domains, it has been speculated that the large concave surface of the TLR ectodomain serves as the ligand binding site for the TLRs. For example, Ribonuclease inhibitor (which binds both ribonuclease A and angiogenin), Internalin A, and RanGAP have demonstrated that the concave surface of the solenoid serves as the primary ligand binding site for these receptors (Kobe 1996, Papageorgiou 1997, Schubert 2002, Seewald 2002). Insertions within the LRR repeats have also been demonstrated to facilitate ligand binding such as those within RanGAP and the CD42b-von Willebrand factor A1.

That TLRs also contain characteristic insertions following the $10^{th}$ and $15^{th}$ positions of the consensus LRR sequence offers evidence of the location of potential ligand binding sites. These insertions have been suggested to be crucial for PAMP recognition. Insertions at position 10 have been speculated to lie in proximity to the

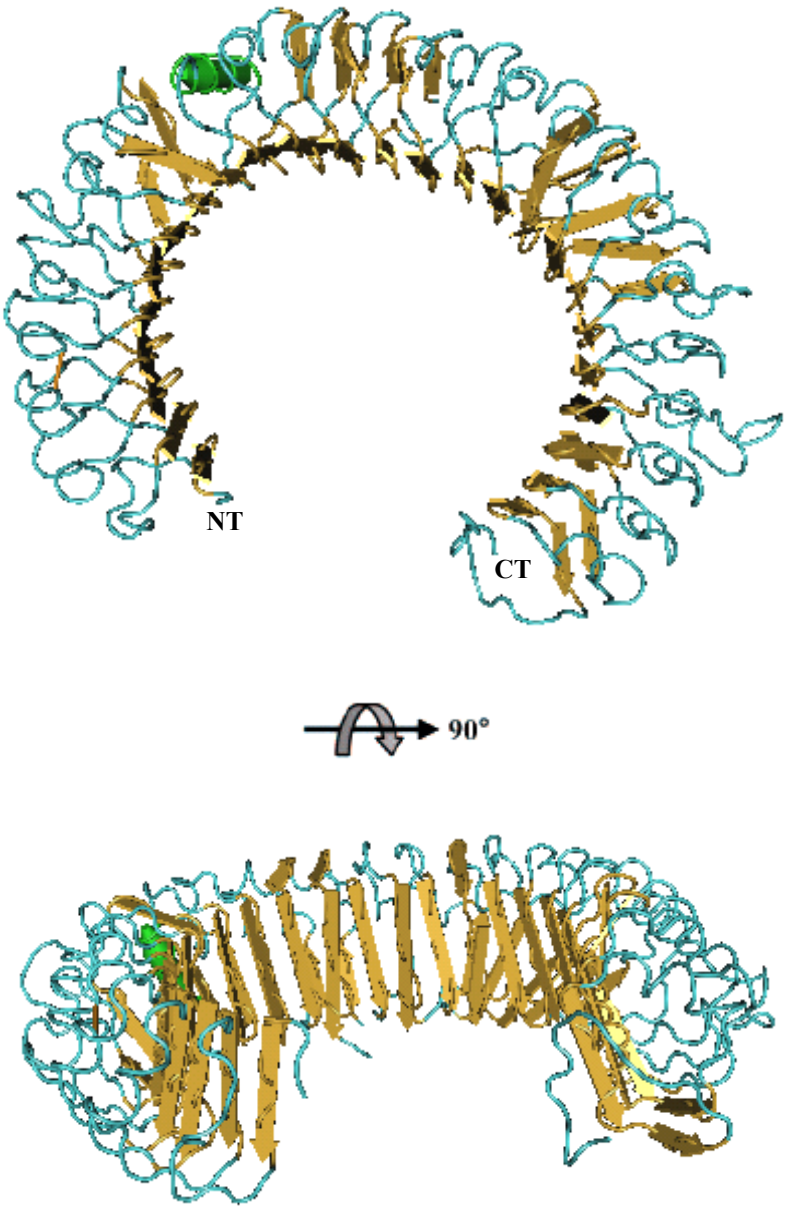



**Figure 1.1: The LRR domain of TLR3 highlighting the overall solenoid shape.** Human TLR3 ectodomain structure (Protein Databank Code 2A0Z) generated with Cn3D version 4.1. Adapted from Bell 2005.

beta-sheet whereas insertions after residue 15 would be predicted to create binding sites at the convex surface (West 2006). Sequence comparisons of the LRR motifs of TLR 7, -8, and -9 demonstrate insertions at position 10 of LRRs 2, 5, 8, and 11 (Bell 2003). These insertions would be expected to occur on the concave face of the receptor and, in the case of TLR9, might create a potential CpG DNA binding site. As a testament to this, the insertion in LRR8 contains two cysteine-rich CXXC motifs, previously shown to mediate the direct binding of CpG-DNA by a transcriptional activator termed CpG-binding protein (Lee 2001). Lending further support for the importance of the insertions to ligand binding, the prospective flagellin binding site on TLR5 (residues 386-407) resides in LRR14 and contains a six-residue insertion after position 15 (Mizel 2003). Analyses such as these have been suggestive of a central role for the concave surface of the TLR ectodomains in ligand binding and a potential role for insertions at positions 10 and 15 within the LRRs of these domains in this regard.

### 1.3.3 TLR Signaling

The conservation of the cytoplasmic Toll/IL-1R (TIR) domain amongst TLR family members results in some commonality in the mechanism of signal transduction. The TIR domain is a protein-protein interaction module that is typically ~200 amino acids in length. Sequence conservation among TIR domains is typically in the range of 20-30% and is suggestive of both sequence and structural diversity (Xu 2000). The highest regions of homology are confined to three short motifs (box 1, box 2, and box 3) of conserved sequences (Janssens 2003, Slack 2000). The importance of these three conserved boxes is underscored by the observation that mutations within boxes 1 and 2

result in the inactivation of signal transduction (Slack 2000). Polotrak has also demonstrated that a point mutation, residue P712, in box 2 of the TLR4 TIR domain results in LPS hyporesponsiveness (Poltorak 1998). Mice with the P712 mutation are unable to recruit downstream effectors, such as Myeloid differentiation primary response protein 88 (MyD88) (West 2006).

The signaling pathway initiated by TLR activation may follow either a MyD88-independent (TLR3) or MyD88-dependent (all other TLRs) signaling pathway but as the work presented in this thesis is based exclusively on TLR9 only the latter will be discussed. MyD88 is a cytoplasmic effector protein that is critical for induction of NF-kB following TLR activation. The presence of a C-terminal TIR domain in the effector enables interactions with the TIR domains of the TLRs. As a testament to the central role of MyD88, MyD88-deficient cells do not respond to peptidoglycans, flagellin, CpG-DNA, or ssRNA (Adachi, 1998, Beutler 2005, Takeda 2003, Yarovinsky 2005).

The binding interaction between a TLR and ligand induces a conformational change within TIR domain, leading to the recruitment of MyD88 to the TIR domain. This interaction leads to the recruitment of the serine/threonine IRAK-4, which contains a death domain, allowing for the interaction with the N-terminal death domain of MyD88.

Following IRAK-4 recruitment, the kinase IRAK-1 is recruited to the complex and is activated following phosphorylation by IRAK-4, resulting in IRAK-1 autophosphorylation. Following IRAK-1 autophosphorylation, Tumor necrosis factor receptor-associated factor-6 (TRAF6) is recruited to the MyD88/IRAK-4/IRAK-1 complex. As a consequence, IRAK-1 and TRAF6 dissociate from the complex with

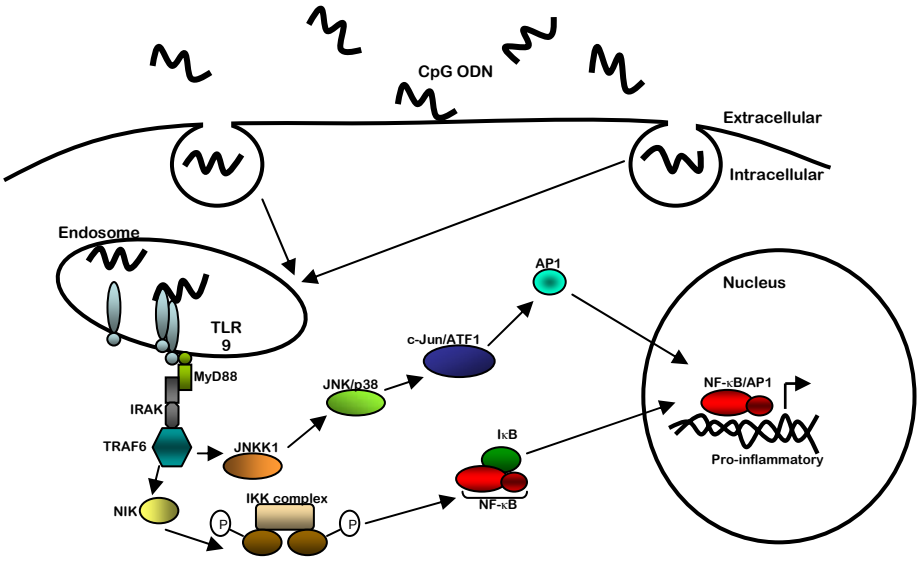



**Figure 1.2: Signal Transduction Associated with Activation of Toll-like Receptor 9.** Binding of bacterial DNA or CpG ODNs promotes activation of TLR9 through dimerization. The ligand-bound dimer recruits MyD88 to the membrane and initiates association with TOLLIP and IRAK4. MyD88 activates IRAK4 to phosphorylate IRAK1. Once phosphorylated IRAK1 recruits TRAF6 to activate TAK1, resulting in activation of the MAPK signaling cascades, JNK1 and p38. TRAF6 also activated the IKKs which phosphorylate IκB. Phosphorylated IκB dissociates from NFκB and is degraded. NFκB translocates to the nucleus to induce expression of pro-inflammatory cytokines, alone or in conjuncture with JNK1 and p38. The resulting pattern of gene expression induces several immunomodulatory effects including cell-mediated immunity, apoptosis as well as direct antimicrobial activity. Adapted from Klinman 2004.

subsequent activation of c-Jun N-terminal kinase (JNK) and inhibitor of κB kinase (IKK). As a consequence of this activator protein-1 (AP-1) and NF-kB are activated, ultimately leading to the transcription of genes encoding proinflammatory cytokines and chemokines (TNF-α, IL-6, IL-8, and IL-1B) and those that produce a $T_H1$-biased immune milieu (IFN-γ, and IL-12) (Klinman 1996).

### 1.4 Activation of TLRs through Dimerization:

Receptor dimerization is a recurrent theme amongst the type I transmembrane receptors (Lemmon 1994). It has been proposed that dimerization of Toll results in the formation of a structurally symmetrical active signaling complex with equivalent ligand binding sites on each monomer (Weber 2005). A similar mechanism has been suggested for the TLR family (Weber 2005). The formation of higher order structures has been demonstrated as a prerequisite for activation of a number of TLRs and likely represents a conserved mechanism of TLR activation. As mentioned previously, heterodimerization has been observed between TLR2, and TLR1 or TLR6 resulting in an increased range of ligands recognized by these receptors thus decreasing the need for additional PRRs (Haijar 2001). Homodimerization has also been observed in TLRs such as TLR3, TLR4 and TLR8 as a requirement for receptor activation (Gangloff 2004).

An investigation by Gangloff demonstrated that the basic mechanism of activation within the TLR family is stimulus-induced crosslinking or oligomerization (Gangloff 2004). Although the authors suggested that the crosslinking of two TLR4 molecules is sufficient for receptor activation, this does not hold true for all TLRs. Investigations of TLR3 dimerization have demonstrated that the crosslinking of TLR3

dimers in the absence of ligand is insufficient for receptor activation (Matsumoto 2002). However, crosslinking of chimeric TLR3 dimers (the TLR3 ectodomain coupled to the CD32a cytosolic domain) with poly(I-C) results in rapid induction of $Ca^{2+}$ mobilization (de Bouteiller 2005). These results suggest that the activation of TLR3 requires conformational changes within TLR3 dimers that are dependent upon ligand binding (de Bouteiller 2005). Thus, the formation of TLR dimers is not the defining step leading to activation, at least for a subset of the TLRs. This likely reflects a prerequisite for activating structural alterations that are specifically induced upon the binding of activating ligands.

The activation of Toll by Spätzle has been reported to involve ligand-induced receptor dimerization and formation of a symmetrical heterotrimeric complex (Weber 2003). The authors speculated that conformational changes within the Toll dimers following ligand binding are required for activation of the complex. As full-length Toll ectodomains form inactive unstable dimers in solution in the absence of ligand, the formation of the dimeric Toll-Spätzle complex is both necessary and sufficient for Toll activation (Weber 2003). Recently, Gay has proposed that the correct orientation of the crosslinked domains is the crucial step for receptor activation in Toll (Gay 2006). Based upon the homology between the Toll and TLR families it has been proposed that the formation of inactive ligand-free dimers, and ligand-induced conformational changes within receptor dimers prior to activation, also extends to the TLR family.

The potential for multiple structural variations of particular TLRs, including monomers, inactive dimers and active dimers, raises the potential for regulation of signaling activity through modulation of the equilibrium between the different forms of the receptor.

## 1.5 A Model of Toll Binding

The investigations of Toll-Spätzle interactions by Weber have culminated with a proposed model of ligand binding and receptor activation for Toll (Weber 2003, Weber 2005). Previously, the authors demonstrated that the stoichiometry of the active signaling complex involved one Spätzle dimer and two Toll monomers. Building on these observations, the authors demonstrated that the binding of a single Spätzle ligand to a Toll ectodomain monomer has a higher binding affinity than the second binding event involving formation of the activated dimeric Toll- Spätzle complex.

Thus, the binding of Spätzle and the formation of an active signaling complex involves two non-equivalent steps (**Figure 4.1, p. 164**). In the absence of ligand, Toll exists in equilibrium between inactive monomers and dimers. The formation of the ligand-free Toll dimer is proposed to involve mediation by the first block of LRRs (Gay 2006). This first block prevents the strong dimer contacts of the second block of LRRs and provides steric constraint against the formation of active ligand-free Toll dimers (Gay 2006). In the presence of Spätzle, the inactive dimer complexes dissociate to form inactive monomeric Toll-Spätzle complexes. Following this, ligand-free Toll monomers are recruited to the Toll-Spätzle complexes with reformation of Toll dimers crosslinked by Spätzle. Promotion of the dimeric Toll-Spätzle complex to an activated form likely results from conformational rearrangements between the Toll monomers. That the second binding event involving the formation of the activated Toll complex is of lower affinity than the initial binding of Spätzle to Toll monomers indicates negative cooperativity.

As a consequence of negative cooperativity, the effective range of ligand required to achieve complete activation of the receptor is dramatically increased. Based

on Michaelis-Menten kinetics, in the absence of cooperativity, an 81-fold increase in substrate concentration is required for complete receptor activation. In the event of negative cooperativity, 400-fold increases in substrate concentration are required to achieve the same activation (Koshland 1996). Consistent with the model of negative cooperativity proposed by Weber, it has been demonstrated that an approximately 300-500 fold change in Spätzle concentrations is required to increase Toll activation from 10% to 90% (Weber 2005). That the ligand concentrations required to reach 90% maximum capacity of signaling activity are very broad has been suggested to increase the ability of the cell to produce differential patterns of gene expression in response to a stimulus (Gay 2006). A similar phenomenon has also been noted for the FSH receptor (Urizar 2005).

### 1.6 Structural Information Regarding the TLR-Ligand Interaction

Although a paucity of structural information is available regarding the interactions between TLRs and their ligands, two independent crystallographic determinations of TLR3 have been reported (Bell 2005, Choe 2005). Both groups have reported the conservation of the horseshoe-shaped solenoid structure and curvature which is common amongst proteins with LRR domains. It was also noted that one face of the solenoid was dominated by heavy glycosylation whereas the other face was glycosylation-free. Although Choe and Bell have reached consensus on the overall three-dimensional conformation of the receptor, they have proposed contrasting dsRNA binding sites.

The model presented by Choe (**Figure 1.3**) suggests that although the inner diameter of the concave surface is sufficient for dsRNA, ligand binding would be compromised due to structural restraints and electrostatic repulsion (Choe 2005). The authors suggested that the glycosylation and the overall negative charge found on the concave surface of TLR3 would conflict with the binding of dsRNA. This negative charge would result in significant repulsion to the negatively charged phosphate backbone of the RNA. Choe instead suggests that the receptor uses the glycosylation-free convex face for ligand binding. In agreement with this proposition, the large glycosylation-free face of TLR3 provides many basic residues to accommodate the negative charge of the phosphate backbone. In addition, a 10-residue insertion within LRR12 also lies on the glycosylation-free face and is in close proximity to this positive region. Notably, LRR12 and LRR20 of TLR3 contain insertions longer than 5 residues; LRR12 has a 10-residue insertion that points towards the glycosylation-free side face and the 11-residue insertion of LRR20 points towards the outer convex surface (Choe 2005). The authors note that although a large proportion of the residues from this insertion were disordered in the structure, they could potentially interact with the major groove of dsRNA. Thus, TLR3 monomers could 'sandwich' dsRNA by positioning of the positive regions of the glycosylation-free faces and the 10-residue insertion of LRR12 on either side of the dsRNA molecule. The authors also propose that TLR3 might form inactive dimers in solution based on the symmetry-related interactions of the receptor in the crystal structure. Thus, the binding of dsRNA to a pre-existing dimer may either strengthen receptor dimerization or induce conformational changes leading to

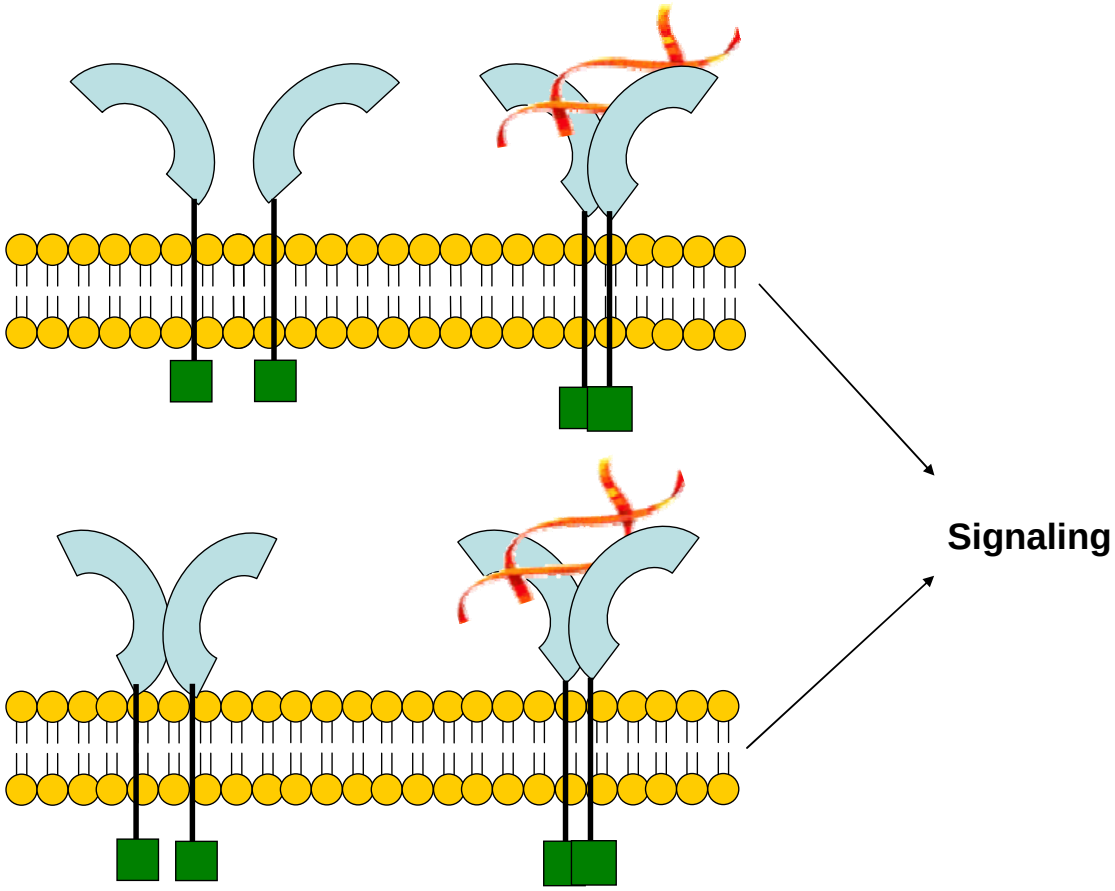



**Figure 1.3: Choe model of TLR3 ligand binding.** dsRNA (orange helix) binding near the N-terminus of the TLR3 ectodomain can induce receptor dimerization to form the active signaling complex or the ligand can bind to preformed inactive dimers. Adapted from Choe 2005.

activation of the receptor, such as has been proposed for Toll (Weber 2005). A similar mode of action has also been proposed for the erythropoietin receptor (Livnah 1999). Although the binding of ligand to the convex surface of TLR3 contrasts with the recurrent use of the concave surface for ligand binding by other proteins with LRR domains, several of these proteins can use sites secondary to the concave surface for ligand binding (Kobe 2001).

In contrast to the Choe model, Bell has suggested that dsRNA may bind within the concave surface of the receptor (**Figure 1.4**) (Bell 2005). The authors noted the presence of sulfate ions at the side chains of several amino acids within the concave surface. These were bound to the side chains of His, Asn, Tyr and Arg residues by hydrogen bonds and were postulated to mimic the phosphate backbone of nucleic acids (Bell 2005). Although Choe suggested that the glycosylations found within the concave surface would impede such an interaction, Bell suggested that the use of insect cells rather than human cells for expression of the recombinant protein might account for the differences in glycosylation patterns. Decreased glycosylation along the concave surface would reduce the steric constraint to dsRNA binding (Bell 2005). It is also noted in the Bell structure that a shallow groove on the glycosylation-free, convex face produced by the two insertions from LRR12 and LRR20 might also act as a dsRNA binding pocket for dsRNA. The authors speculate that this might represent a mode of oligomerization whereby TLR3 ectodomains would bind to the two "faces" of the RNA duplex (Bell 2005). A subsequent investigation demonstrated that deletion of LRR20, but not LRR12, had a dramatic effect of TLR3 activity providing evidence that dsRNA interacts with TLR3 on the glycan-free face of LRR20 (Bell 2006).

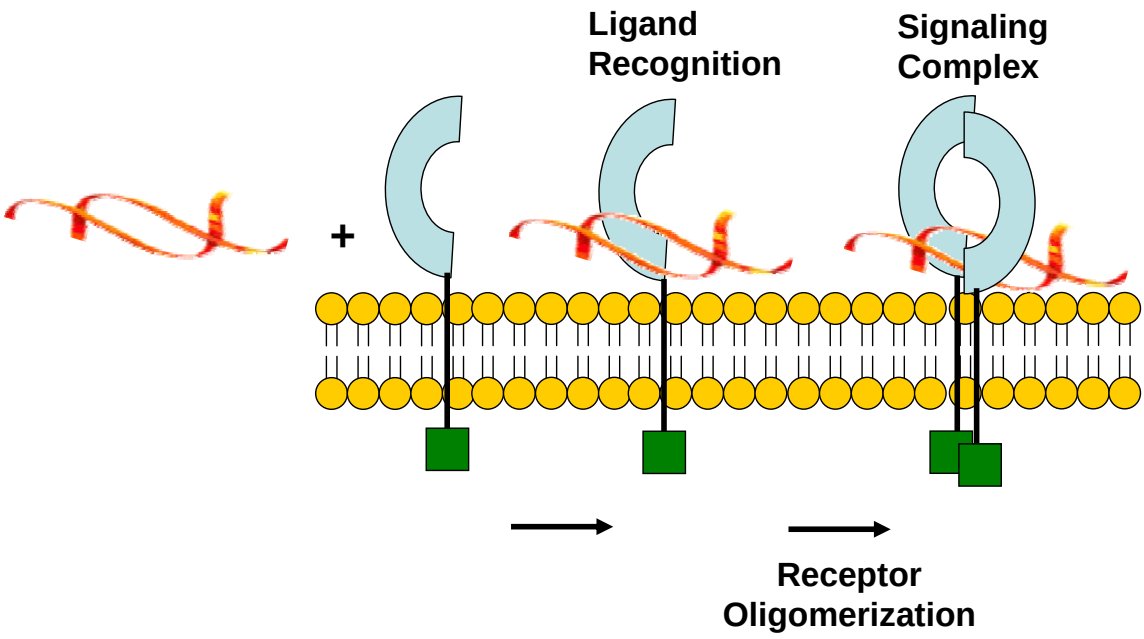



**Figure 1.4: Bell model of TLR3 ligand binding.** dsRNA (orange helix) binds near the C-terminus of the TLR3 ectodomain and induces receptor dimerization to form the active signaling complex. Adapted from Bell 2005.

Thus, rather than resolving the structural mechanisms of nucleic acid recognition, these structures add further controversy by proposing two conflicting models of ligand binding. Choe has proposed the existence of pre-formed dimers that could interact without ligand and, upon ligand binding, would move through a conformational change leading to receptor activation (Choe 2005). In contrast, Bell has proposed that dimerization of TLR3 is induced following ligand binding (Bell 2005).

Adding further controversy, Ranjith-Kumar has demonstrated that deletion of loop 1 in LRR12 is not essential for TLR3 function (Ranjith-Kumar 2007). In agreement with the observations by Bell, a charged surface characterized by an asparagine-rich region from LRR17 to LRR20 was demonstrated to be critical for TLR3 function (Bell 2005, Ranjith-Kumar 2007). The authors also note that the putative RNA-binding region and TLR3 dimerization domain are in close proximity to each other and suggests overlapping activities between these two regions. The observations reported by Ranjith-Kumar are supportive of both the Choe and Bell models. In support of the Choe model, pre-formed dimers are observed in the absence of ligand. In contrast, the specific residues postulated to be involved in RNA-binding support the model proposed by Bell. Thus, rather than resolving the discrepancy of the Choe and Bell structures, the results of Ranjith-Kumar have further fueled the debate.

Recently, Gibbard has investigated the conserved structural features of human TLR8, that together with TLR3, -7, and -9 is localized intracellularly and recognizes microbial nucleic acids as its endogenous ligand (Gibbard 2006). Notably, TLR7, 8, and 9 are more closely related to each other than any other TLRs and thus structural features within the extracellular domain are conserved. Therefore, the elucidation of a particular structural feature in any one of these three TLRs would likely represent a conserved

moiety across all three TLRs. Indeed, TLR7, 8, and 9 share a characteristic 16 amino acid insert, containing four conserved cysteine residues, in LRR8 that is critical for TLR8 mediated signaling in response to ligand; mutation of any of the four conserved cysteine residues in this short insert sequence abrogates TLR8 signaling (Gibbard 2006). This was compounded by the demonstration that a single acidic residue, D543, in LRR17 was also critical for receptor activation by ligand. Interestingly, this residue is conserved amongst TLR7, 8, and 9 and has previously been demonstrated by Rutz to be critical for binding of CpG-DNA by TLR9 *in vitro* (Rutz 2004).

### 1.7 TLR9 and Ligand Recognition

Much of what is known regarding the activation of TLR9 by microbial DNA can be attributed to key observations made over the last two decades. In 1984, Tokunaga demonstrated that mycobacteria DNA fractions with high GC content possessed strong antitumor activity in mice (Tokunaga 1984). A subsequent investigation revealed that short, single-stranded synthetic oligonucleotides with CpG palindromic sequences could induce immune responses, highlighting the importance of this particular PAMP (Tokunaga 1992). Adding further support for the role of CpG sequences in the induction of immune response, poly-(dC,dG) from purified bacterial DNA, but not vertebrate DNA, were demonstrated to induce murine B cell proliferation (Messina 1991). Modification of the cytosine residues within these CpG sequences by methylation abated their immunostimulatory activity (Messina 1993). The relevance of the unmethylated CpG dinucleotide and relation to the mitogenicity of microbial DNA would not be revealed until the elegant work of Krieg and colleagues.

Krieg deduced that the mitogenicity of bacterial DNA might be explained by unmethylated CpG dinucleotides within microbial genomes, a pattern virtually absent in eukaryotic host organisms due to suppression of frequency and modification (Bird 1987, Krieg 1995). This hypothesis was corroborated by the observation that methylation of bacterial DNA with CpG methylase abated mitogenicity as did inversion of the CpG to GpC. Although the CpG dinucleotide represents the minimal element of microbial DNA that triggered immune cell activation, the authors demonstrated that stimulatory activity could be increased or decreased based on a higher order hexanucleotide sequence containing a central CpG. Indeed, human TLR9 knockout cells can be made responsive to synthetic CpG sequences in a species-specific fashion following transfection with mouse TLR9, and vice versa (Bauer 2001). The authors speculate that this reflects preferential binding of CpG motifs by TLR9 in a sequence- and species-specific manner. Activation in mouse and rabbit immune cells is highest when the CpG dinucleotide is flanked by two 5' purines (preferably GA) and two 3' pyrimidines (preferably TC or TT) (Krieg 1995, Yi 1998A, Rankin 2001) whereas the optimal motif in human and bovine cells is GTCGTT, a motif that also appears to be optimal for other vertebrate species such as sheep, cat, dog, goat, horse, pig, and chicken (Hartmann 2000, Brown 1998, Rankin 2001).

Although much of the early work on CpG motifs was suggestive of a potential role for the Toll-like receptors in the recognition of microbial DNA, a direct link between the receptor and PAMP had not been identified. The identification of TLR9 by Hemmi suggested that this newly identified member of the TLR family utilized microbial DNA as an endogenous ligand based on the CpG motif; wild-type mouse splenocytes exhibited proliferative responses to CpG-DNA, but not non-CpG-DNA, in a

dose-dependent fashion whereas TLR9-/- splenocytes did not proliferate in response to either stimulus (Hemmi 2000).

Krieg identified the unmethylated CpG dinucleotde as the discriminating factor between host and foreign DNA. Although CpGs are present in prokaryotes at the expected 1:16 frequency, eukaryotes mask these dinucleotides by the suppressing the frequency of these sequences by as much as 100-fold (Wagner 1994). Eukaryotes also modify the cytosines of CpG sequences by methylation (Krieg 1995). The methylation of DNA in mammals provides a method for suppression of gene transcription. The modification stabilizes chromatin in an inactive configuration as gene expression is inhibited by DNA hypermethylation (Sekigawa 2006). In mammalian cells, enzymes such as DNA methyltransferases (Richardson 1999), methyl-CpG binding protein (Nakao 2001), and histone-modifying enzymes (Sekigawa 2003) methylate the 5-position of deoxycytidine residues to form deoxymethylcytosine. Highlighting the importance of DNA methylation within eukaryotes, 60% to 90% of all CpG sequences within mammalian genomes are methylated and are normally clustered within the CpG-rich CpG islands of gene promoters (Richardson 1999). It has been speculated that the CpG sequences of CpG islands, which are predominantly unmethylated, can initiate innate immune responses upon apoptotic release of nucleosomes (Sekigawa 2006). From the perspective of the higher order CpG motif, the immunostimulatory activity of the unmethylated CpG dinucleotides in vertebrate DNA is suppressed as a result of the propensity for a 5'C and a 3'G surrounding the CpG (Han 1994).

In contrast to the central role of unmethylated CpG motifs in the activation of innate immune responses by TLR9, it has been demonstrated that ODNs devoid of CpG motifs are able to activate innate immune responses in a TLR9-dependent manner.

Although this would appear to contradict the postulate that TLR9 has evolved to specifically recognize unmethylated CpG motifs, it has been considered that TLR9 may have evolved to recognize general DNA structural abnormalities or modifications as well unmethylated CpG motifs (Ishii 2005).

### 1.7.1 Oligodeoxynucleotides as TLR9 Ligands

The identification of the CpG motif as the activating-factor of TLR9 has led to the development of synthetic oligodeoxynucleotides (ODNs) that selectively trigger TLR9-mediated innate immune responses (Krieg 1995). As potential therapeutic agonists of TLR9, ODNs have a considerable advantage over bacterial DNA due to simplicity of manufacturing, ease of sequence alterations, low developmental cost and chemical stability. Optimally, ODNs are 8-30 bases in length with the most potent ODN usually containing two or more CpG motifs (Krieg 2002). As the phosphodiester backbone of native DNA is rapidly digested by serum and cellular nucleases, *in vivo* applications of ODNs have typically adopted a phosphothioate-modified (PTO) backbone.

The PTO backbone (**Figure 1.5**) imparts nuclease resistance due to the replacement of one of the non-bridging oxygen atoms with sulfur. This nuclease-resistant moiety has been shown to improve the half-life of modified ODNs, with an approximate 200-fold increase in CpG-induced B-cell activation (Krieg 1995), as well as to increase their avidity for cell membranes and cellular uptake (Zhao 1993, Zhao 1994, Crooke 1998). The modification is not completely neutral, however, as PTO-ODNs exhibit non-specific binding to a wide variety of proteins (Stein 1993, Stein 1994).

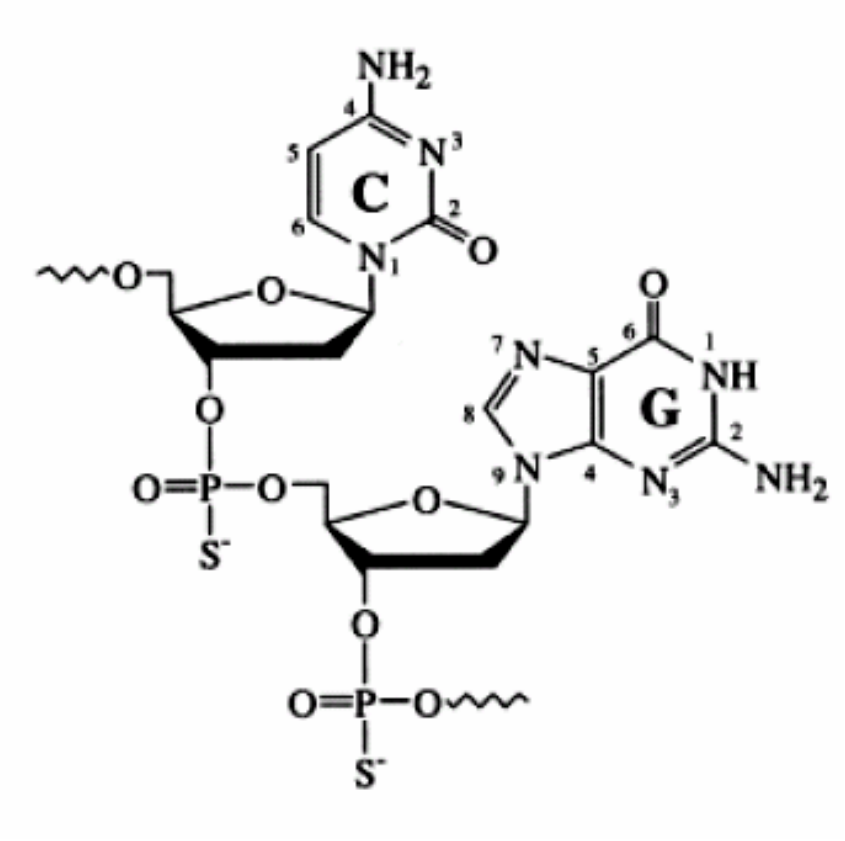



**Figure 1.5: Chemical Structure of a phosphothioate CpG.** Structure generated with ChemDraw Standard Version 10.0.

PTO-modified ODNs have also demonstrated sequence-independent activities such as transcription factor activation (Perez 1994) and non-specific immune stimulation (Monteith 1997).

It should also be noted that unlike PD-ODNs, PTO-modification introduces a chiral center to the DNA backbone. Thus, each internucleotide PTO linkage can be of either Rp or Sp absolute configuration. The question of stereospecificity with respect to the interaction of PTO-ODNs with proteins has been investigated (Krieg 2002). Snake venom phosphodiesterase and human 3'-exonuclease demonstrate preference for the Rp stereoisomer (Burgers 1979, Tang 1995, Koziolkiewicz 1997). Thus, all Sp-PTO-ODNs are more stable intracellularly than their all-Rp counterparts (Krieg 2002). An investigation by Gilar has speculated that the increased nuclease resistance of PTO-ODNs may be related to increased non-specific adsorption of the PTO-ODN to the nuclease enzyme (Gilar 1998). The stability of the Rp stereoisomer was 300-fold less than those with the S-configuration which invites speculation that the Sp stereoisomer is better able to adsorb to the enzyme. In contrast to the aspect of intracellular stability, the Rp stereoisomer appears to more effectively interact with TLR9 (Krieg 2002). Indeed, PTO-CpG-ODNs with the R-configuration demonstrate much greater immunostimulatory activity than their S-configuration counterparts, although cellular uptake rates are identical for both (Krieg 2002).

### 1.7.2 Classes of ODNs

Three classes of ODNs, which differ in their sequences and in the presence and

| ODN Type | Example | Structural Characteristics | Associated Activity |
|---|---|---|---|
| A | GG**TGCAT*CG*ATGCA**GGGGGG | PD and PTO (underlined) backbone. Single CpG motif (italics). Poly G Tail Hairpin. forming sequences (bold) | Stimulate pDCs to release IFN-α. IFN-α mediated APC maturation |
| B | TCCATGGA*CG*TTCCTGAG*CG*TT | PTO(underlined) backbone. Multiple CpG motifs (italics) | Maturation of pDC and TNF production, B-cell proliferation and Il-6 production |
| C | T**CGTCGTT*CG*AACGA*CG***TTGAT | PTO(underlined) backbone. Multiple CpG motifs (italics). Hairpin forming sequences (bold) | Stimulate pDCs to release IFN-α. B-cell proliferation and Il-6 production |

**Table 1.1: Representative Sequences, Structures and Biological Actions of the Different ODN Classes**. Table adapted from Klinman 2004.

location of backbone modifications, have been defined on the basis of their ability to elicit unique cellular responses (**Table 1.1**).

A-Class ODNs (CpG-A), also referred to as type D, are structurally typified by a central phosphodiester region, with one or more CpG motifs in a self-complementary palindrome and poly-G sequences of three or more guanines at the 5' and/or 3' ends. These poly-G sequences result in the ability of the A-class ODNs to form stable higher-order G-tetrads (Krieg 2006). These ODNs are especially potent at inducing IFNα production by pDCs. IFNα production results in the subsequent activation of natural killer (NK) cells and IFN-γ secretion (Krug 2001).

B-Class CpG ODNs (CpG-B), or type K ODNs, are potent B cell activators resulting in increased Major Histocompatibility Class II complex (MHCII) expression, secretion of immunoglobins and B cell proliferation (Krieg 2002). Structurally, CpG-B ODNs have a completely phosphothioate backbone and are typically contained in a linear conformation (Krieg 2006).

Finally, C-Class CpG ODNs (CpG-C), induce both A-Class and B-Class signaling effects (Marshall 2003). CpG-C ODNs are comprised of an entirely phosphothioate backbone with one or more 5' CpG motifs and a 3' palindrome that is thought to induce duplex formation within the endosome (Krieg 2006).

It has been suggested that the differences in signaling between the CpG ODN classes may be due to differential cellular trafficking in terms of uptake and retention (Honda 2005, Latz 2004, Gursel 2002). Differences in intracellular localization patterns between the A- and B-class CpG ODNs have been demonstrated by Gursel (Gursel 2002). The authors noted that the uptake of CpG-A ODNs exceeded that of CpG-B ODNs and that the two classes occupied discrete areas within the same cells with limited

co-localization (Gursel 2002). Notably, this difference in localization patterns was directly related to the ability of CpG-A to form G-tetrads as control ODN with a poly-G tail had similar distribution patterns. Class-specific responses suggest that although all CpG ODNs can interact with TLR9, there are likely differences in the mechanism of trafficking, recognition and/or signal transduction induced by each class of ODN either due to the sequence/structure of ODN or differences in accessory proteins or co-stimulatory molecules that are recruited to the TLR9-ODN complex which has not yet been identified.

A recent investigation suggests that the different ODN classes induce different post-TLR9 signaling pathways leading to the induction of different transcription factors (O'Neill 2006). This is also consistent with the discovery of secondary signaling pathways that have been identified for other Toll-like receptors involving unique adaptors and IRF transcription factors (Oshiumi 2003a, Oshiumi 2003b).

### 1.7.3 TLR9 and Sequence Specificity

A common benchmark often employed to demonstrate the specificity of CpG activation is negative control ODNs in which the activating dinucleotide is reversed to a GpC (GpC-ODNs). A wealth of cell stimulation experiments demonstrates the preferential ability for CpG, rather than GpC-ODNs, to initiate innate immune responses. This biological specificity is generally assumed to reflect sequence-specific binding by TLR9. However, ligand binding investigations have reached contradictory conclusions with respect to the ability of TLR9 to discriminate nucleic acids in a sequence-specific fashion.

**SPR Analysis**

Most of the ligand binding investigations of TLR9 employ surface plasmon resonance (SPR) technology (Figure 1.6). SPR analysis is based on the reflection of light by thin metal films, typically gold or silver. At a specific angle, the energy from the incident photons interacts with delocalized electrons within the metal film (plasmons) resulting in total attenuation of the light. The angle at which this occurs is dependent on the refractive index of the interface at the two sides of the metal surface. Target molecules are immobilized on the back of the metal surface and addressed by ligands, which are suspended in a mobile phase running along a flow cell. If binding occurs between the ligand and immobilized target, the local refractive index will change, leading to changes in the SPR angle. These changes to the SPR angle are detected as changes in the intensity of the reflected light. The magnitude of the SPR signal change is directly proportional to the immobilized mass and can be interpreted in terms of the stoichiometry of the interaction.

Arguing in favor of sequence-specific binding by TLR9, investigations by Rutz (Rutz 2004) and Cornelie (Cornelie 2004) utilized SPR analysis to investigate ligand binding by a recombinant form of the receptor. Investigations by Rutz, utilizing immobilized PD-ODNs, noted that the recombinant murine TLR9 ectodomain exhibited sequence-dependent binding of PD-ODNs with the most favorable interactions occuring at acidic pH, reflective of the endosomal environment (Rutz 2004). These results support the *in vivo* demonstrations that ODNs, in a sequence-dependent manner, undergo favorable interactions with TLR9 within the endosome.

In contrast, but equally convincing, is an investigation by Latz in which CpG and

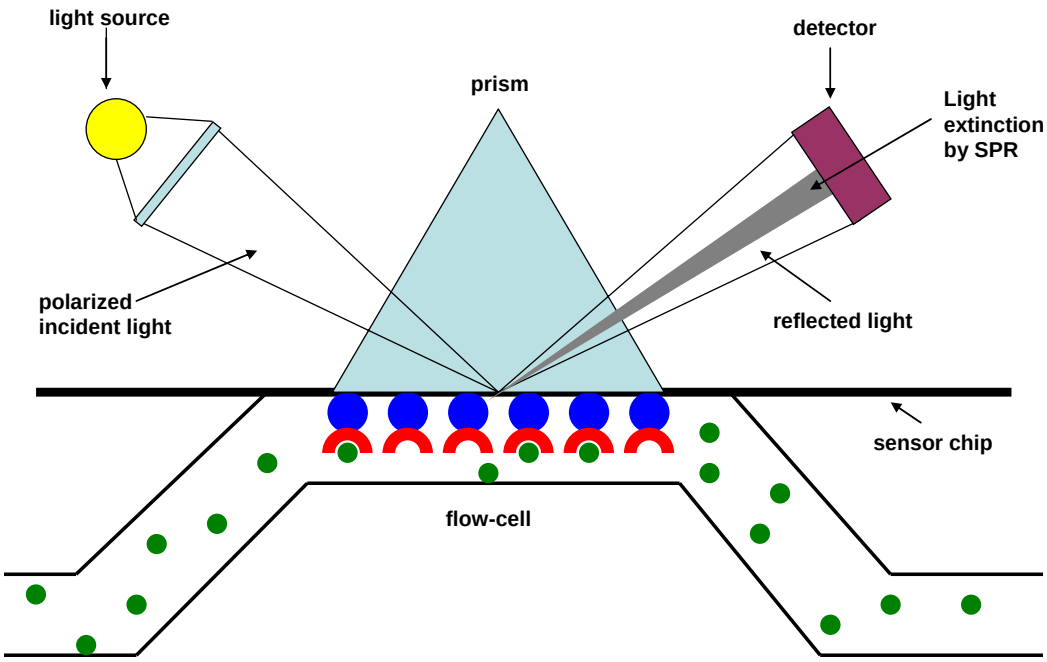



**Figure 1.6: SPR analysis.** Target molecules (blue and red) are attached to the underside of the sensor chip and may interact with ligands (green circles) suspended in the mobile phase.

GpC-containing ODNs were shown to be equally effective in mediating co-immunoprecipitation of TLR9 from cellular extracts, supportive of the physiological interaction of the receptor with nucleic acids in a sequence-independent fashion. That GpC-ODNs can inhibit CpG-mediated cellular activation was suggested by the authors to represent competition between CpG- and GpC-ODNs for the same binding sites on TLR9 (Latz 2004).

Adding further controversy to the specificity of binding by TLR9, Rutz demonstrated that the ability of TLR9 to discriminate higher order species-specific CpG motifs was limited to PD-ODNs (Rutz 2004). PTO-ODNs were found to be indiscriminate in their ability to bind TLR9, and also demonstrated non-specific binding to other TLR family members (Rutz 2004). In direct contrast, Roberts demonstrated through functional assays that PTO-modified ODNs were associated with higher sequence specificity and that the ability for cells to discriminate higher-order CpG motifs was dependent upon the PTO-modification (Roberts 2005). They, and others, have suggested that the ability for different sequences to preferentially activate TLR9 in a species-specific fashion is an artifact of the PTO-modification (Yasuda 2006, Roberts 2005).

Clearly the PTO-modification is not a neutral substitution, although it is not clear whether the consequence is to increase or decrease the specificity of interaction with TLR9. PTO-ODNs have been described as “sticky” with the tendency to interact with other proteins, including other Toll-like receptors (Rutz 2004). The interactions with proteins other than TLR9 may account for the ability of PTO-ODNs to exert CpG-independent side effects including induction of B cell proliferation, splenomegaly, and tissue infiltration by mononuclear cells (Baek 2001, Khaled 1996, Monteith 1997).

Collectively, the differences between natural and PTO-ODNs are perhaps best summarized by the observation that "the rules governing TLR9 activation by PTO-ODN and PD-ODN differ from each other" (Yasuda 2006).

These results appear to contradict the proposed sequence-dependency of TLR9 activation. The observations by Roberts, however, suggest that sequence-dependency is limited to PTO-ODNs and cannot be recapitulated with PD-ODNs (Roberts 2005). This is supported by the observations of Yasuda and Rutz that appear to resolve the discrepancies between TLR9 sequence-dependency in regards to PTO- and PD-ODNs (Yasuda 2005, Rutz 2004). An investigation by Yasuda demonstrated that the enforced endosomal translocation of vertebrate DNA, via 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) coupling resulted in TLR9 activation (Yasuda 2005). In contrast to the dogma of CpG motif-specific TLR9 activation, these observations suggest that the limiting factor for TLR9 activation may be endosomal translocation efficiency rather than the binding proclivities of the receptor. Recent investigations by Yasuda support this argument. High concentrations of DOTAP-coupled PTO-GpC-ODNs have immunostimulatory activity and PD-ODNs coupled to DOTAP activated TLR9 in a sequence-independent fashion (Yasuda 2006). These results were also corroborated by SPR analysis and introduced a significant caveat from previous analyses; DOTAP-coupling results in the endosomal translocation of up to 10-fold more ODN than the natural endocytic pathway. Thus, although SPR analysis demonstrated that at equimolar concentrations, PD-CpG-ODN bound to TLR9 with approximately 3-fold greater efficiency than that of the non-activating motif, this sequence-dependent binding could be abolished by increasing the concentration of PD-GpC-ODN. This was reasoned by the authors to be representative of the "low-affinity" binding of TLR9 to the PD-ODNs

and that this low affinity could be overcome by simple introduction of a CpG motif or, alternatively, by increasing the concentration of PD-GpC-ODN. Thus, ligands that would normally be regarded as "low-affinity" ligands, such as GpC-ODNs, upon enhanced endosomal translocation have the propensity to activate TLR9 once an undefined threshold concentration is exceeded.

Thus, the relationship between DNA binding and TLR9 activation remains controversial; PTO-ODNs are able to bind TLR9 in a sequence-independent fashion whereas natural PD-ODNs and plasmid DNA containing CpG sequences have higher affinities to TLR9 than non-CpG ODNs or methylated plasmid DNA although this can be overcome by increasing the functional concentration of the non-optimal ligands. This would represent a unique deviation from the accepted TLR9 DNA ligand paradigm; the species- and sequence-specific activation of TLR9 is confined to those molecules that are translocated to the endosome through the natural uptake route (Yasuda 2005).

### 1.7.4 Single- and Double-Stranded TLR9 Ligands

Investigation of TLR9 activation by cell stimulation assays have been biased to the use of single-stranded ODNs. The ultimate challenge to PRRs is to discriminate self from non-self and a differentiating factor between eukaryotic and microbial organisms is the presence of plasmid DNA. Thus, it is intriguing to speculate that TLR9 might possess the ability to bind DNA of single- and double-stranded nature in both linear and plasmid forms. Indeed, previous investigations have noted that the initiation of innate immune responses by plasmid DNA is suggestive of TLR9 activation (Sawamura 2005,

Spies 2003, Sato 1996) and that the stability of plasmid DNA within the endosome would allow for such an interaction (Bennett, 1985).

Investigations of single- and double-stranded DNA ligand recognition by TLR9 have reached opposing conclusions. Cornelie has demonstrated TLR9-dependent induction of NF-kB in response to plasmid DNA and activation is abrogated by plasmid methylation or chloroquine administration (Cornelie 2004). SPR analysis demonstrated that recombinant TLR9 bound plasmid DNA effectively in a methylation-dependent and sequence-dependent fashion. Competition experiments demonstrated that CpG-ODNs, but not GpC-ODNs, can compete for TLR9 binding with plasmid DNA (Cornelie 2004). All of these observations support plasmids as TLR9 ligands. In contrast, Rutz has demonstrated very weak binding of double-stranded linear ODNs by recombinant TLR9 and minimal NF-kB induction (Rutz 2004). Furthermore, an investigation of the immunostimulatory activities of plasmid DNA and single- and double-stranded ODNs by Zelenay reported similar observations (Zelenay 2003). Plasmid DNA expresses adjuvanticity as a single- or double-stranded moiety whereas the immunostimulatory activity of CpG-ODNs is reduced when double-stranded. Although the plasmid binding results of Rutz contradict those of Cornelie it should be noted that the nature of the double-stranded ligand in the two analyses differs. Notably, the Cornelie investigation utilized plasmid DNA whereas the investigation by Rutz employed linear double-stranded ODNs.

Thus, the investigations by Rutz and Cornelie, although contrasting in regards to TLR9 activation by single- or double-stranded DNA molecules, would be presumed to

reflect the types of DNA released within the endosome following bacterial phagocytosis and thus the types of DNA ligands that could potentially interact with TLR9.

### 1.7.5 Localization of TLR9 and Endogenous DNA

That the PAMP recognized by TLR9 is also an endogenous biomolecule presents a unique problem for TLR9 in host-pathogen DNA discrimination. The activation of TLR9 has been proposed to be based on unmethylated CpG motifs present within microbial DNA. Although such sequences are rare within eukaryotes, host DNA does have the potential to activate TLR9. As host genome possesses the potential to activate unnecessary innate immune responses, a number of protection mechanisms appear to limit the potential for this interaction.

One of the primary mechanisms by which the cell has evolved to limit innate immune activation by host nucleic acids is by the intracellular localization of nucleic acid binding TLRs. Prior to CpG stimulation, TLR9 resides within the endoplasmic reticulum and upon CpG internalization, is rapidly translocated to the endosome (Leifer 2004). The authors also demonstrated that this translocation event might involve a direct pathway between the ER and the endosome as TLR9 remains sensitive to EndoH, an enzyme that digests immature N-linked sugars added in the ER but modified within the Golgi. Biotinylation studies have demonstrated that following stimulation with CpG-DNA, a small fraction of TLR9 becomes accessible to the extracellular environment suggesting ER-to-endosome fusion (Latz 2004). Although this would suggest that the localization of TLR9 acts as a safety mechanism to limit access to host CpG motifs, it does not define the nature of the interaction between TLR9 and host

DNA. Using a chimeric TLR9 that localized to the cell surface, Barton demonstrated that cells transfected with TLR9 or the surface-exposed TLR9 variant responded equivalently to CpG ODN stimulation at a broad concentration range of the ligand (Barton 2006). However, stimulation with chromatin-containing immune complexes or purified vertebrate genomic DNA resulted in selective activation of the chimeric TLR9 variant. The immunostimulatory effect of the genomic DNA was negated when pre-incubated with DNase-I (Barton 2006). Thus, it is evident that the localization of endogenous TLR9 prevents activation by self-DNA. In a similar manner it has been demonstrated that endosomally translocated self-DNA can activate TLR9 while extracellular self-DNA cannot (Yasuda 2006). This suggests either specificity in the uptake of the DNA, with the selective endocytosis potentially limiting exposure of TLR9 to inappropriate ligands, or that self-DNA is degraded before it has the opportunity to serve as a TLR9 ligand.

Activation of TLR9 by self DNA may serve a physiological function to initiate localized inflammation in response to damaged or dying cells. Accumulating evidence suggests that host DNA has TLR9-activating potential and thus presentation of host nucleic acids might allow for induction of innate immune responses (Vallin 1999, Marshak-Rothstein 2004, Boule 2004). Recently, Ishii and Akira have suggested that the culmination of this phenomenon might be that TLR9 has evolved to detect not only the CpG motif within pathogenic organisms, but also some molecular pattern within host-derived DNA, albeit a CpG motif as of yet unidentified nature or some other conserved motif (Ishii 2005).

The potential for immunostimulation by host DNA is minimized due to cellular compartmentalization as DNA is normally sequestered within the nucleus or

mitochondria. This is not the case, however, with damaged cells as nucleic acids may be released into the cytoplasm or extracellular environments. It has been suggested that DNA released in the situations may lead to activation of innate immune responses (Ishii 2005). Thus, although the sub-cellular localization of TLR9 serves to protect the host from the immunostimulatory activity of self-nucleic acids, this does not remove the potential of self-DNA to bind to and activate TLR9. As a secondary protective mechanism, host DNA minimizes the potential for TLR9 activation through the presence of suppressive DNA sequences. These sequences have been found to selectively block CpG-mediated immune stimulation by suppressive motifs rich in poly-G of GC sequences, or optimally the sequences found within the telomeric regions of mammalian DNA (the repeat TTAGGG) (Gursel 2003).

The mechanisms for limiting TLR9 activation by host DNA highlight two important considerations for design of TLR9 immunotherapeutics. Firstly, it would be anticipated that the interaction between TLR9 and its ligand would have evolved to be optimized for physiological environment in which they occur. As the late endosome provides a unique chemical environment with an acidic pH, changes in the protonation state of either the receptors or ligands may be prerequisite for formation of an activated complex. Secondly, that TLR9 has the ability to be influenced functionally by inhibitory nucleic acid sequences implies the receptor has the ability to bind both stimulating and non-stimulating sequences, highlighting the functional distinction between ligand binding and receptor activation. This would be of particular consequence as it limits the potential to discover novel agonists through screening assays based solely on their ability to interact with the receptor.

### 1.7.6 TLR9 and Systemic Auto-immune Disease

As mentioned in the previous section, self-DNA immunoglobulin G (IgG) complexes have been demonstrated to contribute to the pathogenesis of systemic auto-immune diseases. Indeed, self-DNA-IgG complexes contribute to the pathogenesis of systemic auto-immune diseases such as systemic lupus erythematosus (SLE) and have been demonstrated to activate innate immunity in a TLR9-dependent fashion (Leifer 2006, Ishii 2005). Systemic auto-immune diseases refer to diseases in which pathogenesis is attributed to the production of auto-antibodies specific to a varied, but limited, set of self-biomolecules. As many of these self-determinants are derived from macromolecular structures with similar features to PAMPs, such as chromatin (Rifkin 2005), self-tolerance may be lost. From the perspective of TLR9, these host biomolecules may interact with the receptor due to of structural conservation between host and pathogen DNA as a consequence of the limited number of unmethylated CpG dinucleotides found within the vertebrate genome.

It has been suggested that the onset of systemic auto-immune disease and the process of apoptosis are intricately linked in that apoptotic bodies not rapidly cleared from the system could potentially serve as a source of auto-antigen. As a testament to this, many of the recurring auto-antibody targets in diseases such as SLE and scleroderma are protein/DNA or protein/RNA complexes (Rifkin 2005). This represents the specific paradox of TLR9 activation by self-DNA; how is the physical interaction between receptor and ligand mediated and why do the security measures found in mammalian DNA not reduce TLR9 activation?

Several lines of evidence suggest that abnormal DNA methylation is a key determinant in SLE. Indeed, DNA methylation has been implicated in the development of several diseases (Sekigawa 2006). For example, DNA hypomethylation within proto-oncogenes has been reported to increase expression of proto-oncogenes, potentially promoting malignant transformation, and has also been demonstrated to increase mutation rates (Laird 1994, Chen 1998). CpG island methylation has been associated with conditions such as retinoblastoma (Laird 1994). From the perspective of TLR9, the chromatin isolated from apoptotic or dying cells exhibits modifications such as aberrant DNA methylation patterns. Huck has observed an increased release of nucleosomes enriched in hypomethylated CpG-rich DNA due to accelerated apoptosis rates and defective apoptotic cell clearance in SLE patients (Huck 1999). It has also been demonstrated that therapeutics for drug-induced lupus inhibit the action of DNA methyltransferases (Cornacchia 1988). Huck has demonstrated that over 90% of patients treated with procainamide, an anti-arrhythmic therapeutic, develop anti-nuclear antibodies with approximately 20% exhibiting lupus-like symptoms (Huck 1999). This is supported by reports that SLE patient sera are enriched in hypomethylated CpG-rich low molecular weight DNA (Sano 1989, Krapf 1989, Corvetta 1991). These examples are supportive of a role for TLR9 in autoimmune disorders such as SLE.

The physical boundary between TLR9 and the extracellular matrix has been regarded as one of the primary defense mechanisms limiting the activation of TLR9 by host DNA. It has been proposed that the interaction between DNA-IgG complexes and cellular receptors might promote the delivery of host DNA to the endosome in patients with SLE (Marshak-Rothstein 2006). Indeed, the Fcγ receptor (FcγR), specific for the Fc portion of IgG on pDCs, is required for detection of immune complexes; pretreatment

of SLE patients with FcγR blocking agents ablates the ability of pDCs to be stimulated by sera containing immune complexes (Bave 2003). Thus, receptor-mediated endocytosis of DNA-immune complexes in patients with systemic auto-immune disease represents one of the limiting factors for TLR9 activation. It has also been proposed that the DNA within immune complexes in cases of systemic auto-immune disease might differ from normal mammalian DNA; DNA isolated from immune complexes is often enriched in CG content as (Sano 1982). They have demonstrated that DNA extracted from T cells of SLE patients is hypomethylated compared to that of normal T cells. The efficient removal of DNA from sera might also represent a limiting factor for the development of DNA-immune complexes. Nucleases such as DNase-I, the primary nuclease within serum, have been correlated with the suppression of SLE-like auto-immune diseases development. DNase-I KO mice demonstrate symptoms of SLE-like auto-immune diseases and DNase-I mutations have been observed in SLE patients (Napirei 2000, Yasutomo 2001).

Although the precise mechanism by which the interaction between TLR9 and host DNA is mediated has yet to be determined, treatments have thus far focused on the inhibition of the interaction. Anti-malarial agents such as chloroquine have been used therapeutically for more than a century to treat SLE and have recently been demonstrated to interfere with the binding of CpG-DNA by TLR9 (Marshak-Rothstein 2006, Rutz 2004).

### 1.7.7 pH Dependence of TLR9 Ligand Binding

It has been demonstrated that TLR9 and CpG DNA co-localize within the endosomal compartment and that this is an essential component for cellular activation (Ahmad-Nejad 2002, Rutz 2004). As such, compounds such as bafilomycin A1, a specific inhibitor of the endosomal and lysosomal V-type ATPase, and chloroquine, a weak base that perturbs endosomal pH by partitioning into the organelle, interfere with endosomal acidification and inhibit CpG-driven TLR9 signaling (Hacker 1998, Yi 1998, Yoshimori 1991, Macfarlane 1998).

Previously, an investigation by Rutz demonstrated that the recombinant extracellular domain of TLR9 binds CpG-DNA in a pH-dependent manner. Binding between the receptor and CpG-DNA was highest at the acidic pH of the endosome (pH4.5-6.5) and was drastically reduced at neutral pH (7.4) (Rutz 2004). The authors noted that chloroquine and its analog quinacrine, inhibited the binding between the receptor and CpG-DNA in a dose-dependent fashion.

The dependence on acidic pH for ligand binding has also been demonstrated for other endosomal TLRs. Ligand binding and receptor dimerization were demonstrated to be more efficient at acidic pH for TLR3 and TLR8 (de Boutellier 2005, Ranjith-Kumar 2007, Gibbard 2006). de Boutellier demonstrated that the activation of TLR3 by poly(I-C), and the induction of receptor dimerization by the ligand, could be effectively blocked by bafilomycin A1 and chloroquine (de Bouteiller 2005). Optimal responsiveness of TLR3 to poly(I-C) requires acidic pH and progressively decreased towards neutral pH, at which receptor activation was completely ablated (de Boutellier

2005). Similarly, Gibbard demonstrated that the activation of TLR8 is highly pH-dependent and is abolished by bafilomycin A1 (Gibbard 2006). The pH-dependency of TLR8 signaling was speculated by the authors to be largely due to the influence of pH on receptor homodimerization. Ranjith-Kumar has reported similar observations and demonstrated that TLR8 can form inactive dimers in the absence of ligand at acidic pH (Ranjith-Kumar 2007).

The pH dependence exhibited by the interaction between TLR9 and DNA has been postulated to be the result of two potential, but not mutually exclusive, mechanisms (Gibbard 2006). Firstly, the acidic environment within the endosome might induce conformational changes in the receptor that either increase the ligand-binding proclivities of TLR9 or provoke receptor dimerization to an activated complex. The authors speculate that this may result from the ionization of histidine sidechains within the receptor ectodomain; TLR7, 8, and 9 have a conserved histidine residue in LRR18. A conceptually similar, but functionally inverted, mechanism to regulate ligand binding during intracellular trafficking has been characterized for the low density lipoprotein receptor. In this example binding of the low density lipoprotein ligand occurs at neutral pH at the cell surface. During endosomal acidification, changes in the protonation state of a pseudoligand of the ectodomain displace the ligand (Rudenko 2002).

Alternatively, changes in the protonation state of the ligand might promote ligand binding by the receptor. The ionization of nucleic acids, and in particular cytodine residues of the CpG repeat, may change during the endosome maturation. The pKa for cytodine in 4.2; however, the pKa is likely considerably higher when the base is found within the context of a polynucleotide due to the phosphate backbone and would result in a positively charged ligand. In support of this hypothesis, the mutation of

ectodomain residue Asp-543 in TLR9, resulting in loss of a negatively charged region within the receptor ectodomain, ablates TLR9 ligand binding (Rutz 2004). It is hypothesized that TLR9 activation may therefore involve binding of the positively charged CpG ligand to negatively charged regions of the receptor ectodomain (Gibbard 2006). Based on these observations, the potential may exist for the design of more effective TLR9 agonists through the incorporation of modifications that influence the protonation state, and thereby ligand efficiency, of nucleic acids.

## 1.8 Therapeutic Applications of ODNs

As oligodeoxynucleotides are easy to manufacture, highly stable, and of low manufacturing cost, they are attractive as therapeutic modulators of TLR9 activation. Supporting the efficacy of synthetic ODN administrations, there has been minimal concern regarding the systemic toxicity of either the PD- or PTO-backbone ODNs. Previous investigations for anti-sense and aptamer therapeutics can be applied to the therapeutic applications of TLR9 modulators as all are oligodeoxynucleotide molecules. Extensive characterizations of ODNs in terms of absorption, distribution, metabolism, and elimination properties have been completed over the past decade (Geary 1997, Levin 2001). These investigations have noted that synthetic PTO-ODNs exhibit rapid clearance into tissues but do not show signs of crossing the blood-testis (which restricts cytotoxic agents from passing into the seminiferous tubules) or blood-brain barriers. Sequence-independent activities of PTO-ODNs related to the phosphothioate backbone have also been characterized. Chronic dosing of PTO-ODNs in rodents noted high local ODN concentrations in the kidneys following repeated high concentration ODN doses

with the potential for induction of degenerative lesions and necrosis in the proximal tubules (Levin 2001, Jason 2004). In contrast, investigations of human renal function in relation to PTO-ODN administration have reported no adverse effects. Thus, a species-specific bias may exist in the prediction of PTO-ODN toxicity. That TLR9 expression in rodent immune cells is much broader than that of primates has been suggested as a potential mechanism for this phenomenon (Krieg 2006).

Following the demonstration that ODNs containing CpG motifs could selectively activate TLR9, half a dozen TLR9 agonists have been taken into human clinical trials, including two in Phase III trials (Krieg 2006). These applications have focused on four primary areas of development: 1) infectious disease prevention and therapy, 2) as vaccine adjuvants, 3) treatments for the reduction of allergic responses, and 4) in cancer therapies. As a testament to the potential of these novel TLR9 agonists as future therapeutics, CpG-ODNs have been administered in over a dozen clinical trials. The vast majority of Phase I studies have focused on the immunomodulatory activities of CpG-ODNs when administered alone, or in combination with vaccines, passive immunization, or allergens. Currently, two controlled Phase III human clinical trials are underway (initiated in 2005 by Pfizer) for the class B CpG-ODN PF-3512676 (Adis Intern. Lim. 2006). These trials are evaluating PF-3512676 administration in combination with chemotherapy for treatment of patients with advanced non-small-cell lung cancer (NSCLC). Phase II clinical trials demonstrated promising results as response rates were significantly improved from 19% in patients undergoing standard chemotherapy treatment to 37% in patients who also received PF-3512676 (Krieg 2006). Further supporting the application of TLR9 agonists as novel therapeutics, a Phase IIa clinical study of a class C CpG-ODN, CPG 10101 (Coley) investigating its activity as a

monotherapy against hepatitis C virus (HCV) is in preparative stages following a Phase 1b trial that demonstrated up to a 1.6 mean maximum log reduction of viral RNA with ODN administration (Krieg 2006). That inappropriate TLR9 activation may contribute to the pathogenesis of disorders such as SLE and rheumatoid arthritis suggests TLR9 antagonist therapeutics may also be beneficial. Indeed, pre-treatment of mice with suppressive ODN significantly reduced CpG-induced inflammatory arthritis in mice (Zeuner 2003). These examples highlight the promise of TLR9 agonists and antagonists as future therapeutics.

The activation of TLR9 by CpG-ODNs has the potential for deleterious side affects, in particular the potential to trigger autoimmune disorders. It has been demonstrated that CpG-ODN treatments can aggravate auto-immune disorders in mouse models of lupus (Hasegawa 2003), multiple sclerosis (Ichikawa 2002), colitis (Obermeier 2002), and arthritis (Ronaghy 2002). Normal mice have also proven susceptible to the deleterious effects of TLR9 over-stimulation as the administration of high does of bacterial DNA led to the development of auto-antibodies against double-stranded DNA (Gilkeson 1993). Concerns have also been raised regarding the ability of CpG-ODN therapies to facilitate the development of toxic shock by lowering the pathological threshold of lipopolysaccharide-tolerance. Co-administration of CpG-ODNs with sub-lethal doses of LPS and D-galactosamine resulted in severe mortality and morbidity (Sparwasser 1997, Cowdery 1996, Hartmann 1996). However investigations in mice using weekly administrations of CpG-ODNs over a four month period demonstrated no adverse health effects. Similarly, therapeutic administrations of high dosages of antisense ODNs (synthetic ODNs that are complimentary to the sense sequence of a particular mRNA) to patients with Crohn's disease had no adverse effects

(Yacyshyn 2002). Thus, it would seem that although no major toxic effects are observed upon administration of DNA alone, the presence of multiple TLR agonists may contribute to the pathogenesis of sepsis based on the combined sensitization of each agonist.

Therapeutic development of novel TLR9 modulators has been hindered by the paucity of information regarding the direct interaction between the receptor and ligand. The advent of a convenient screening assay would allow for the evaluation of TLR9 agonists and antagonists based on a combination of the direct characterization of the receptor-ligand interaction and on the basis of the cellular responses they elicit rather than the current biased approach that utilizes only cell stimulation results. This bias is inherently dangerous as the numerous points of regulation within the TLR9-mediated cell signaling pathway are reduced to a single output thus removing the ability to discriminate specific events from the overall biological process. The danger of this approach is that the reduction of numerous potential points of regulation to a single output does not permit discrimination of specific events, such as ligand binding, from the overall biological process.

## 1.9 Host Defense (cationic) Antimicrobial Peptides

### 1.9.1 General Characteristics of Host Defense Peptides

Host defense antimicrobial peptides (HDPs) are ubiquitous defense biomolecules found in virtually all forms of life - including bacteria, fungi, plants, invertebrates, and vertebrate species (Jenssen 2006). Currently more than 900 different HDPs have been

identified or predicted from nucleic acid sequences (http://www.bbcm.univ.trieste.it/~tossi/amsdb.html). That HDPs have been retained within the innate immune system during the co-evolution of eukaryotic hosts and prokaryotic pathogens suggests that they play a pivotal role in the innate immune response. As lower organisms lack adaptive immunity, these peptides form a central component of the innate immune system against pathogen invasion. Although the vertebrate immune system has both innate and adaptive immune components, HDPs still serve an essential role in infection resolution. This is highlighted by the observation that genetic disorders such as specific granule deficiency syndrome (Ganz 1988), atopic dermatitis (Ong 2002), and morbus Kostmann syndrome (Putsep 2002) increase the frequency and severity of bacterial and viral infections within the host. As the primary role of HDPs in lower organisms is the resolution of microbial infection, these peptides have traditionally been characterized based on their antimicrobial activities (McPhee 2005). Recently, in has become clear that they also play a pivotal role in the regulation of mammalian immunity, thus the title of "host defense peptides" rather than "antimicrobial peptides".

Cationic HDPs are gene-encoded and are normally produced as inactive precursor peptides. Peptide activation follows proteolytic cleavage of the pre-peptide region. As such, HDPs are normally defined based on a set of criteria: they are short (10-50 amino acids, generally <10 kDa), an overall positive charge (+2 to +10) with high concentrations of lysine and/or arginine residues, and a high proportion of hydrophobic residues (≥30%) (McPhee 2005).

### 1.9.2 Host Defense Peptide Families

As host defense peptides typically exhibit minimal sequence similarity, they are often grouped according to the major structural conformation adopted in the membrane-associated state. It should be noted however that the structural class given has little, if any, relationship to its biological activity (McPhee 2005).

The amphipathic alpha-helical class of HDPs is the most abundant and well characterized class of HDPs. Upon interaction with the hydrophobic membrane environment this class of HDPs adopts an amphipathic alpha-helical conformation with one helical face containing the majority of the hydrophobic residues, the opposite containing a large proportion of the polar residues (McPhee 2005). These peptides are often short (<40 amino acids), devoid of cysteine residues, and found to be unstructured or linear in non-hydrophobic environments. Notable HDPs of this class include the antimicrobial peptide alamethicin (Payne 1970), the food preservative nisin (Van de Ven 1991), the magainins (Zasloff 1987), and the human cathelicidin LL-37.

The beta-stranded cationic peptides are the second largest class of HDPs. These peptides are highly diverse at the level of primary structure. Commonly, the beta-stranded HDP class is comprised of several antiparallel beta-strands with stabilization by one or more disulfide bonds (Yount 2005). Recently, a common motif that integrates all cysteine-containing HDPs has recently been elucidated by multidimensional proteomic analysis. The γ-core, characterized two antiparallel beta-sheets and basic residues along its axis, has been found to recur in all major classes of cysteine-stabilized HDPs, across all biological kingdoms, and may represent a unifying structural motif in HDPs (Yount 2004). The beta-stranded HDP class includes the highly antimicrobial tachyplesins

(Yang 2001) and polyphemusins (Miyata 1989), protegrins (Chen 2000), and the commercialized antibiotic gramicidin S (Gibbs 1998).

The last class of HDPs, the extended peptide class, is defined by the absence of secondary structure (McPhee 2005). These peptides normally contain high proportions of amino acids such as histidine, tryptophan, or proline and tend to adopt an overall extended conformation upon interaction with hydrophobic environments. Examples of peptides belonging to the extended class include indolicidin, a bovine neutrophil peptide, and the porcine peptide fragment, tritpticin (McPhee 2005). These structures are stabilized by hydrogen bonding and van der Waals forces as a result of contact with lipids in contrast to the intra-molecular stabilization forces found in the former peptide classes.

Traditionally, the name "antimicrobial peptide" has been limited to small cationic peptides. The demonstration that larger polypeptides and proteins also possess antimicrobial activity may expand the number of biomolecules falling under this nomenclature. Acid hydrolysis products of lactoferrin, an 80 kDa glycoprotein of the transferrin family, have been demonstrated to have direct antimicrobial activity (Gifford 2005, Hoek 1997). Interestingly, molecules with previously demonstrated immunomodulatory activity have now been found to possess direct antimicrobial activities as well. The microbicidal chemokines, or kinocidins, traditionally regarded as chemoattractive immunomodulatory signaling molecules, have recently been ascribed antimicrobial activity to a specific C-terminal domain of the protein (Bjorstad 2005). The C-terminal domain is similar in structure and composition to many alpha-helical antimicrobial peptides and is typically cationic and amphipathic (Yount 2006).

### 1.9.3 Host Defense Peptide Distribution

Host defense peptides are involved in both intracellular and extracellular defensive responses. HDP synthesis is constitutive or inducible and occurs within the granules of phagocytic cells, or alternatively, HDPs are secreted by epithelial cells (Devine 2002).

In vertebrates, two groups of host defense peptides are prominent, the cathelicidins and the defensins. Cathelicidins, characterized by a conserved N-terminal cathelin domain, are a large and diverse group of HDPs. The significance of the cathelin domain is due to the inactivity of the peptide in the absence of the proteolytic cleavage of this segment to generate the mature peptide (Jenssen 2006). Thus, cathelicidins are naturally stored in an inactive propeptide state and are normally found within the secretory granules of neutrophils, mucosal surfaces in the mouth, lung, and genitourinary tract (Bowdish 2005a). Structurally, the cathelicidins are extremely diverse, with the exception of the N-terminus, and are represented by alpha-helical, beta-hairpin, and proline/arginine-rich peptides. The second prominent mammalian HDP group is the cyclic defensins. In an analogous manner to the cathelicidins, defensins are synthesized as prepeptides and require proteolytic processing to become active; however, the structural diversity of the defensin family has led to their division into three subfamilies: the α-, β-, and θ-defensins. The α- and β-defensins are categorized based on the pattern of disulfide bonding between the six conserved cysteines in these two subfamilies and are widely distributed in vertebrate species; θ-defensins, identified only in Old World monkeys, are subgrouped based on their macrocyclic nature (Jenssen 2006). The defensins, much like their HDP counterparts, the cathelicidins, are normally

found within immune cells (neutrophil granules, macrophages, NK cells) or at epithelial surfaces (respiratory mucosal surfaces, intestinal epithelial cells, and urogenital tract mucosal surface) and their expression may be either constitutive or inducible.

### 1.9.4 Biophysical Properties of Host Defense Peptides

The role of host defense peptides in the innate immune system requires their selective toxicity towards a pathogenic target. Thus, HDPs share unifying characteristics that are quintessential for antimicrobial activity, including catioinicity, amphipathicity, and hydrophobicity. These characteristics produce a selective affinity to microbial determinants that are easily accessible, are conserved across the broad spectrum of microbes, and are relatively immutable (Yount 2005). During the co-evolution of host and pathogen, HDPs development has met these constraints.

#### 1.9.4.1 Catioinicity

The majority of HDPs have a conservation of cationicity with enrichment in lysine and arginine residues. Thus, HDPs carry positive charges that range from +2 to +10, depending on pH and the number of basic residues (Giangaspero 2001). The cationicity of HDPs likely acts as a key determinant for the initial electrostatic interaction with the highly electronegative cell envelopes of bacteria (Giangaspero 2001). Unlike eukaryotic cell membranes, comprised primarily of zwitterionic and neutral membrane lipids, bacterial cell membranes are comprised of highly electronegative phospholipids including phosphatidylglycerol, phosphatidylserine, and

cardiolipin. These components confer an overall negative charge to the microbial cell surface and thus an attractive target for HDPs. Additionally, microbial membranes contain acidic compounds such as LPS (Gram-negative bacteria) and teichoic and teichuronic acids (Gram-positive bacteria) that impart additional negative charge and therefore additional attraction to the cationic HDPs (Yount 2005).

It has also been demonstrated that the transmembrane potentials of prokaryotes are typically 50% greater than their eukaryotic counterparts. This chemiosmotic potential has been proposed to act eletrophoretically on HDPs attached to the microbial surface (Hancock 1997). It should also be noted that although increased HDP cationicity is generally associated with increased antimicrobial activity there are limits to this relationship. Investigations of HDP cationicity have demonstrated that increased cationicity in the magainin HDPs led to an increased hemolytic propensity and loss of antimicrobial activity (Dathe 2002).

#### 1.9.4.2 Amphipathicity

In addition to cationicity, the specificity of interaction between HDPs and the microbial membrane is also influenced by amphipathicity. Traditionally, HDPs form amphipathic structures upon contact with the hydrophobic environment of the microbial membrane. Peptide amphipathicity reflects the polarization and relative proportions of hydrophobic and hydrophilic elements within a peptide. Although many protein conformations can achieve amphipathic structures, the alpha-helix represents one of the simplest. Most alpha-helices have a periodicity of polar and apolar residues of approximately 3-4 residues per turn (Yeaman 2003). The resulting structure, with the

polar side chains aligned along one side of the helix and the apolar residues lining the opposite side, is optimized for interaction with the amphiphilic microbial membrane (Dathe 1999). It has been demonstrated that the amphipathicity of an HDP correlates with both antimicrobial activity and cytoxicity (Yount 2005). However, a high degree of peptide helicity and/or amphipathicity has been correlated with increased toxicity towards the neutral cell membrane of the host (Jelokhani-Niaraki 2000, Lee 2003).

#### 1.9.4.3 Hydrophobicity

The hydrophobicity of an HDP is defined as the proportion of hydrophobic residues within a peptide (Yeaman 2003). Typically, HDPs are comprised of ≥30% hydrophobic residues. As peptide hydrophobicity governs the ability of a peptide to partition into the lipid bilayer, it is an essential requirement for HDP-membrane interactions. As described previously for peptide amphipathicity, increasing HDP hydrophobicity above a specific threshold correlates with increased host cell toxicity and diminished antimicrobial activity (Wieprecht 1997, Tachi 2002). Therefore, hydrophobicity represents another feature of HDPs that has been optimized through evolution to achieve selective toxicity against microbial cell membranes.

### 1.9.5 Targeting the Bacterial Membrane and Self-Promoted Uptake

As discussed in the previous sections, host defense peptides have been optimized to interact with the bacterial membrane. Structurally, the Gram-negative bacterial outer membrane is an asymmetric bilayer, with the inner leaflet dominated by phospholipid

and a highly divergent outer leaflet primarily composed of LPS (McPhee 2005). Due to a high content of phosphates and acidic sugars, LPS molecules carry a negative charge. The juxtaposition of LPS molecules in the microbial membrane creates the potential for significant destabilizing repulsive forces between the LPS molecules. As a stabilization mechanism, divalent cations, such as $Mg^{2+}$ or $Ca^{2+}$, are bridged between LPS molecules to partially neutralize the electrostatic repulsion between the negatively charged groups (Nicas 1980). Thus, the initial interaction between an HDP and the microbial membrane involves the binding of the peptide at the divalent cation binding sites on LPS at the cell surface (McPhee 2005). The affinities of HDPs for LPS have been demonstrated to be at least three orders of magnitude higher than those for divalent cations (Hancock 1997). This displacement of the divalent cation bridges results in local destabilization of the outer membrane. As a consequence, local transient "cracks" are formed within the membrane with subsequent uptake of the HDP through the destabilized membrane culminating in the "self-promoted uptake" of the HDP (McPhee 2005).

#### 1.9.5.1 Attraction

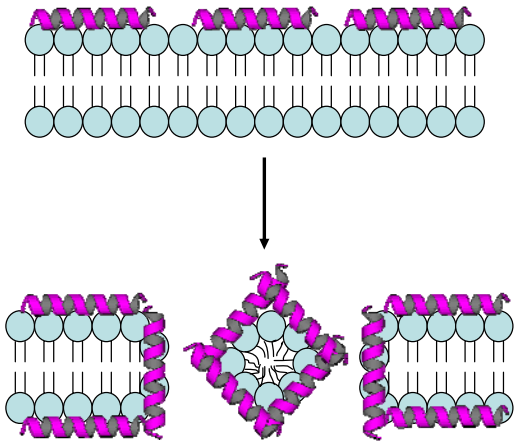
The association of HDPs with lipid bilayers has been observed to be directly related to the ratio of peptide to lipid (Yount 2005). At low peptide/lipid ratios, peptides can be found in oriented parallel to the membrane. As the ratio increases, the peptides reorient themselves perpendicular to the membrane, ultimately inserting into the bilayer. Following membrane insertion transmembrane pores are formed (Yount 2005). The insertion of peptides into the lipid membrane, with the subsequent formation of transmembrane pores, has been described by three models.

#### 1.9.5.2 Membrane Insertion

The barrel-stave model (**Figure 1.7**) describes the insertion of helical peptide bundles or "barrels" in a ring around an aqueous pore (Brogden 2005). The hydrophobic regions of the HDPs are aligned with the lipid core regions and the hydrophilic peptide regions form the interior region of the pore. The "stave" moniker refers to the individual transmembrane spokes within the "barrel". In the second model, a high density of HDPs accumulates at the bilayer surface forming an HDP "carpet" (**Figure 1.7**). During this associative phase the cationic regions of the HDP interact with the anionic phospholipid head groups. As a threshold concentration of peptide is reached, unfavorable membrane energetics result in membrane disruption. In contrast to the barrel-stave model, membrane dissolution occurs in a dispersion-like manner rather than through the formation of transmembrane pores (Yount 2005). The third model, the "torroidal pore" model, is functionally similar to the barrel-model (**Figure 1.7**). Although both models ultimately result in the formation of transmembrane pores, the torroidal pore model differs in the association between the HDPs and the membrane. Rather than forming an amphipathic barrel the HDPs are continuously associated with the polar head groups of the lipids due to the induction of lipid monolayer bending throughout the pore (Brogden 2005). The resulting transmembrane pore is formed by the intercalation of HDPs with lipid and the pore lining formed by the hydrophilic regions of the inserted peptides and the lipid head groups.

Although there is evidence to support all of the above models, it has been suggested that membrane permeabilization may include elements from all three models (Brogden 2005).

A



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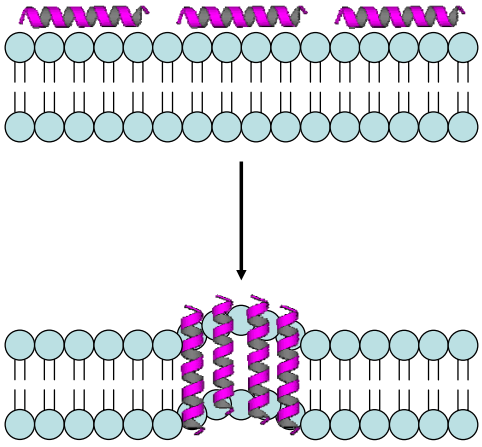

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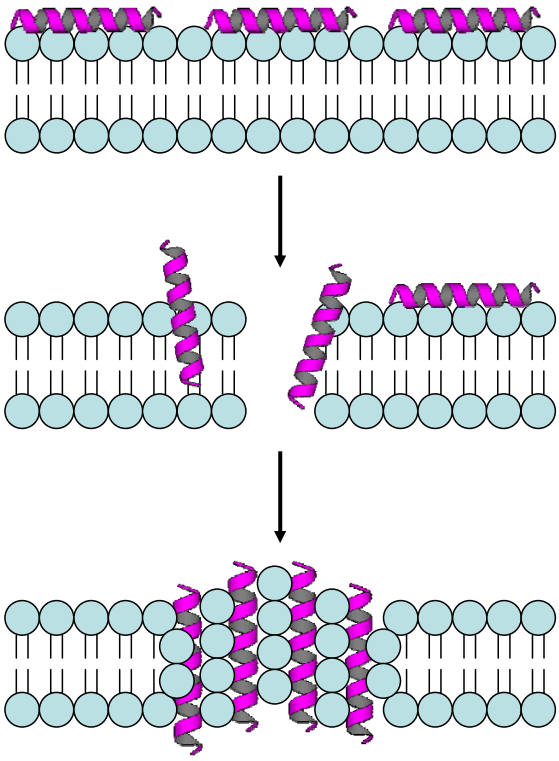


**Figure 1.7: Host Defense Peptide Membrane Insertion Models.** A. The carpet model of host defense peptide-induced killing. B. The barrel-stave model of host defense peptide-induced killing. C. The torroidal model of host defense peptide-induced killing. Adapted from Brogden 2005.

### 1.9.6 Host Defense Peptides and Cell Death

The paradigm for the antimicrobial activity of host defense peptides has long been considered to be a consequence of insurmountable defects in the microbial membrane. Thus, cell death would ensue based on the leakage of ions and metabolites, depolarization, defective biopolymer synthesis, and loss of cellular respiration (Yount 2005). Although it is likely that these effects contribute to cell death, recent observations suggest dissociation between membrane perturbation and microbial death (Yount 2005). Koo has demonstrated that HDP-mediated membrane permeabilization does not invariably result in microbial death (Koo 2001). It has also been demonstrated that depolarization of *Pseudomonas aeruginosa* following gramicidin S exposure does not effectively kill the microbe (Zhang 2000).

Thus, the direct microbicidal activity of HDPs has been speculated to involve perturbation of both extracellular and intracellular architecture. At the bacterial membrane, HDPs have been demonstrated to disrupt peptidoglycan synthesis (Chitnis 1990). In a similar manner, the disruption of intracellular processes as a consequence of HDP exposure has also been observed. Exposure of *Staphylococcus* to HDPs has been demonstrated to inhibit DNA and/or RNA synthesis prior to cell death (Xiong 2002). The authors speculated that this was due to the direct interaction between the cationic HDP and nucleic acids as has been observed for histone proteins. Similarly, the HDP PR-39 has been demonstrated to bind directly to DNA and RNA (Boman 1993). The targeting of intracellular biomolecules by HDPs is not limited to nucleic acids. Kragol has demonstrated that pyrrhocidin, drosocin, and apidaecin (all insect antibacterial peptides) bind to the bacterial heat shock protein DnaK (Kragol 2001). An investigation

by del Castillo has also demonstrated a direct interaction between the HDP microcin B17 and DNA gyrase (del Castillo 2001).

### 1.9.7 Direct Antimicrobial Activity of Host Defense Peptides

Traditionally, host defense peptide investigations have focused on the direct antimicrobial activity of these peptides. Recently however the relevance of the direct microbicidal activity of HDPs *in vivo* has been called into question. Firstly, the physiological concentrations of many HDPs are far lower than those required to exert antimicrobial activity (Bowdish 2005a). HDPs such as LL-37 and the beta-defensins are found in physiological concentrations far lower than those required to exert antimicrobial activity *in vitro* (Bowdish 2005a). Secondly, the physiological niches in which HDPs are normally sequestered would be highly antagonistic to antimicrobial activity. Physiological concentrations of salt and cations have been found to suppress the antimicrobial activities of HDPs (Bowdish 2005a, Friedrich 1999). It should also be noted that a large proportion of HDP antimicrobial activity investigations have used assays that do not directly mimic physiological conditions. Traditionally, antimicrobial activity has been assessed in buffers devoid of physiological concentrations of divalent cations such as $Mg^{2+}$ or $Ca^{2+}$ (1-2mM), monovalent cations such as $Na^{+}$ and $K^{+}$ (100mM), and polyanionic biomolecules such as glycosaminoglycans and mucins (Hancock 2006). Thirdly, that virtually every peptide sequence possesses some degree of antimicrobial activity *in vitro* suggests that this may be a general phenomenon associated with all peptides as opposed to specifically HDPs (Bowdish 2005a).

Although numerous arguments can be made against the physiological relevance of the direct antimicrobial activity of HDPs, there have also been observations that corroborate such a role for these peptides. Indeed, it can be argued that in the appropriate physiological setting HDPs would be expected to have meaningful direct microbicidal activity. The α-defensins present within azurophilic granules of neutrophils have been estimated to reach mg/ml concentrations (Ganz 1987). That HDP genes are subject to positive selection, and belong to the most rapidly evolving group of mammalian proteins, has also been suggested to indicate a physiological role for HDPs in host immune defense (Maxwell 2003, Peschel 2006).

Although arguments for and against a direct antimicrobial activity for HDPs have been raised, it is evident that these peptides play a pivotal role in the innate immune response. The deficiency of α-defensins in human neutrophils can increase susceptibility to recurrent infections (Bowdish 2005). Further supporting a role for HDPs in innate immunity, the deficiency of the human cathelicidin LL-37 and human α-defensins (HNP1-3) has been correlated with frequent oral bacterial infections and severe periodontal disease in morbus Kostmann (Putsep 2002). In rodent models the deficiency of matrilysin, required for the cleavage and activation of murine intestinal α-defensins, led to increased susceptibility to oral *S. typhimurium* challenge in transgenic mice (Wilson 1999).

### 1.9.8 Immunomodulatory Activities of Host Defense Peptides

Recently, the protective functions of host defense peptides have been observed to result partially from activities not related to their microbicidal activity (Finlay 2004).

Rather, HDPs have been demonstrated to modulate the innate immune response. Importantly, these immunomodulatory activities are retained under physiological conditions. As such, it has been speculated that immunomodulation may represent the primary action of these peptides *in vivo* (Bowdish 2005b).

The immunomodulatory activities of HDPs have been attributed to specific roles in the orchestration of innate immune responses. HDPs have been demonstrated to recruit phagocytic cells to the site of infection (Bowdish 2005a). Indeed, α-defensins have been demonstrated to reduce microbial load in mice, an activity that was associated with an influx of phagocytes to the site of infection (Welling 1998). The induction of chemokine synthesis by HDPs has been reported. LL-37 and the defensins have been demonstrated to enhance the release of the chemoattractant IL-8 (Scott 2002, Van Wetering 1997). Angiogenesis stimulation has been observed following exposure to HDPs. LL-37 has been observed to increased endothelial cell proliferation and formation of vessel-like structures (Koczulla 2003). Immunomodulatory activities such as mast cell degranulation promotion, dendritic cell differentiation, nonopsonic phagocytosis promotion, and fibrin clot lysis inhibition have also been reported (McPhee 2005).

In addition to the activities described above, many HDPs have been demonstrated to neutralize the potentially deleterious effects of endotoxin *in vivo* and *in vitro*. During an innate immune response, the induction of excessive inflammatory mediators can lead to tissue damage or sepsis. If left unchecked, this uncontrolled inflammatory response can be lethal (McPhee 2005). Thus, in addition to the immunomodulatory activities associated with HDPs, the ability to suppress LPS-induced

inflammatory response is a favorable activity. Although the direct binding of LPS by HDPs has been observed (Rustici 1993, Gough 1996), recent evidence suggests an alternative mechanism. Peptides such as CEME (also known as MBI-27) and polymyxin B have been observed to neutralize TNF-α synthesis in mice, even when administered 60 minutes following LPS exposure. Similarly, microarray experiments of the interaction of CEMA, a synthetic insect-derived peptide, with LPS demonstrated selective modulation of LPS-induced gene expression (Scott 2002, Tjabringa 2003) adding further support for a non-LPS binding mechanism during endotoxemia neutralization.

The demonstration that anti-endotoxin activities of host defense peptides require relatively high concentrations of peptide has raised concerns regarding host toxicity. Addressing this concern, an investigation by Bowdish demonstrated that the therapeutic administration of multiple peptides at low concentration might resolve these concerns (Bowdish 2005b).

### 1.9.9 Therapeutic Applications of Host Defense Peptides

The therapeutic development of conventional antibiotics has often been biased towards a "single-target, single-action" clinical mechanism. Since their inception more than a half-century ago, antibiotic therapies have been a cornerstone in the battle against infectious disease. However, the extensive application of conventional antibiotics, in addition to the limited introduction of new antibiotic classes, has served as a selective pressure for the emergence of multi-drug resistant microbial strains. This has provoked

the exploration for new antimicrobial agents and strategies. Host defense peptides are regarded as a leading candidate for alternative antimicrobial therapies.

Host defense peptides have significant advantages over conventional antibiotics. In contrast to the single mode of action of conventional antibiotics, HDPs are multi-functional components of the innate immune system. As such, HDPs have demonstrated direct antimicrobial activity, immunomodulatory and/or endotoxin-neutralizing activities, and synergism with conventional antibiotic therapies (Zasloff 2002). Further supporting the therapeutic application of HDPs, these molecules have retained their activities throughout evolution in the face of continual microbial pressure.

As a testament to this, many HDPs are currently advancing through discovery, development and clinical trials. For example, Migenix is investigating the indolicidin-like peptides in the treatment of catheter-associated infections with advancement to Phase IIIb clinical trials (Hancock 2006). Administration of the indolicidin-like MX-226 peptide resulted in a 49% reduction of local catheter site infections and a 21% reduction of catheter colonization (Marr 2006). To date however, only four cationic peptides have advanced into phase 3 clinical trials (Pexiganan, Iseganan, Neuprex, and Omiganan) with only two demonstrating efficacy (Hancock 2006).

Concerns have been raised however regarding the efficacy of HDP therapies. Central to this are concerns that therapeutic applications of HDPs may serve as a selective pressure for the induction of microbial resistance. As these molecules represent a central component of the innate immune system, HDP therapies may have severe consequences if cross-resistance to endogenous HDPs is induced. Concerns have also been raised regarding the cytoxicity of HDP therapies. Thus far, clinical trials of

HDPs have focused on topical application of peptide to address surface infection. As such, there is limited information regarding the potential systemic toxicities associated with HDP treatments. A phase I study of the protegrin analog IB-367 demonstrated no clinically significant adverse effects at peptide concentrations that reduced oral microflora (Mosca 2000). Investigations of the hemolytic activities of HDPs continue to focus on strategies to minimize systemic toxicity concerns for HDPs. Recent demonstrations that antimicrobial peptides share features with eukaryotic nuclear localization signal peptides (Lau 2005), and HDPs can translocate freely into cells (Sandgren 2004), argue for more focus on subtle toxicities that may be associated with HDP therapy.

The pursuit of HDP therapies has also been limited by high costs of peptide production. HDP therapies range in cost from $100 and $600 per gram (the average daily dose for most systemic therapeutics). This contrasts sharply with the low cost of conventional antibiotics (aminoglycosides cost $0.80/gram) (Marr 2006). Thus, the development and testing of large numbers of peptide derivatives has been limited and has created new impetus to create large-scale peptide production platforms. Recently, investigations of have focused on a variety of recombinant DNA methods (using bacterial, fungal, plant, and animal production systems) for the large-scale production of HDPs. Novozyme Inc. has reported the large-scale production of plectasin, a natural fungal peptide, in a proprietary fungal expression system at the scale and purity necessary for therapeutic administrations (Mygind 2005). In addition, reductions in HDP sizes and methods for increasing the systemic stability of HDPs (thus reducing dose quantities and frequency) are also being pursued (Hilpert 2005).

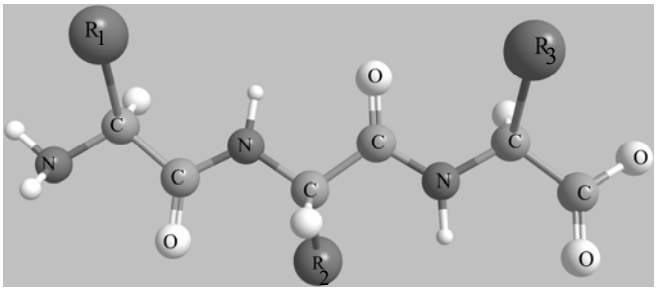
High-throughput screening methods for monitoring HDP activities are also being employed to reduce HDP development costs. Recently, the Hancock laboratory has developed a novel system for the high-throughput generation of HDPs with improved activity which forms the basis for the HDP studies presented in this thesis. Using this screen, the authors investigated the individual substitution of each residue within the synthetic HDP Bac2A with all of the 19 conventional amino acids. This method provides the sensitivity needed to monitor general antimicrobial activities while employing low sample volumes.

That host defense peptides are labile to endogenous protease enzymes limits their therapeutic potential. That basic residues are an obligate feature of HDPs, endogenous trypsin-like enzymes represents a significant therapeutic impediment. HDP are significantly sensitive to proteases with half-lives of minutes *in vivo* (Finlay 2004). Peptides that are resistant to proteases, while retaining the activities of the natural parent peptide, would offer a potential resolution to this problem.

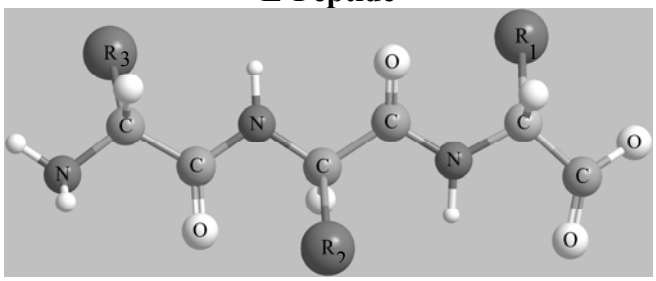
As potential alternatives, all D-amino acid peptides and retro-inverso peptides have been explored as potential HDP variants. Ideally, these modifications decrease the lability of peptides to proteases while retaining the activities of the natural peptide. Investigations by Hamamoto *et al.* and Chen *et al.* have demonstrated that enantiomeric peptide derivatives retain the antimicrobial activity of the natural peptides, are completely resistant to protease treatment, are mirror-images in terms of secondary structures, and display negligible cytotoxicity (Hamamoto 2002, Chen 2006). That the antimicrobial activity of HDPs is retained in the enantiomeric peptide derivative suggests that HDPs do not undergo a stereoselective interaction at the bacterial membrane. Although all D-amino acid HDPs are intriguing for the design of

antimicrobial peptides they are of limited for applications involving the immunomodulatory activities of these peptides. As the immunomodulatory activities of HDPs is linked to receptor-mediated activation of signaling pathways their interaction with chiral moieties is a requirement (Bowdish 2005a). A unique modification has been proposed for peptides that would bestow protease resistance and maintenance of amino acid side chain topology: the retro-inverso modification.

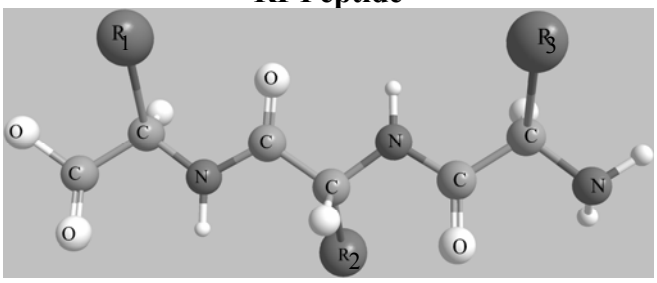
Retro-inverso (RI) peptides are directional isomers of the natural peptide (amino acid assembly in reversed order from that of the natural peptide) with inverted stereochemistry at all alpha carbon chiral centers (**Figure 1.8**) (Fischer 2003). As such, the overall side-chain topology of the RI analogue is analogous to that of the natural peptide and might allow for the accommodation of the peptide derivative in a protein binding site. Reversal of the peptide bond direction inverts the backbone amide bonds resulting in shifted hydrogen bond patterns from that of the parent peptide. This has been speculated to account for the inability of RI derivatives to universally mimic native peptides (Howl 1999, Apletalina 2000, Beglova 2000, Sahu 2000, Warner 2001). RI-modification of peptide hormones has also been investigated and in general the modification is not well tolerated (Fischer 2003). For example, investigations of RI-modified osteogenic growth peptide, which promotes bone anabolism and haematopoieses, demonstrated that the RI-modified peptide lacked biological activity (Bab 2002). In contrast, Snyder has demonstrated that that an RI derivative of p53 can restore endogenous p53 activity; the RI derivate induced apoptosis by activation of endogenous p53 and by restoration of function to several p53 DNA contact mutants (Snyder 2004). RI-modified peptides have also been investigated as potential alternatives for natural protease-sensitive HDPs. Notably, an investigation of an




**L-Peptide**




**RI-Peptide**




**RI-Peptide (rotated 180°)**

**Figure 1.8: Structure of Peptides and their Retroinversed Counterparts.** Retro-inverso peptides present the same three dimensional structures with respect to side chains but have a reversal of the peptide bonds.

RI-derivative of indolicidin (RI-indolicidin) by Nagpal demonstrated that the RI- and natural-peptide had identical functional behaviors; antimicrobial activities and endotoxin binding properties of both peptides were identical (Nagpal 2002). These results would argue that for some HDPs there is at least partial conservation of immunomodulatory activities upon retro-inversion, highlighting the potential of this modification in HDP development for therapeutic applications.

### 1.9.10 Bacterial Host Defense Peptide Resistance

The ability of pathogenic microorganisms to survive host defense peptide exposure results from either constitutive (passive) resistance or inducible (adaptive) resistance.

Constitutive resistance mechanisms, which bestow HDP resistance as a result of ubiquitous expression of resistance factors, have been demonstrated for a variety of bacterial species including *Serratia sp.*, *Proteus sp*., and *Providencia sp.* (Yeaman 2003). These resistance mechanisms are predominantly due to alterations in structural features that decrease HDP attraction. For example, *Staphylococcus aureus* has a unique cell membrane lipid composition with enrichment of unsaturated menaquinones and lysyl-phosphatidylglycerol, a less electronegative derivative of phosphatidylglycerol (Nahaie 1984). Capsule production as a means of combating opsonization and phagocytosis has also been demonstrated in many virulent bacterial and fungal pathogens (Friedrich 1999). It is speculated that capsule production confers HDP resistance as a result of the anionic nature of both the carbohydrate and phosphate moieties of the capsule (Friedrich 1999). It has also been demonstrated that pathogens

may exploit physiological niches as a means of conferring HDP resistance. For example, *P. aeruginosa* preferentially colonizes tissue with abnormal osmotic and ionic strengths that ablate the antimicrobial activity of the HDPs within the particular niche (Yeaman 2003). As HDPs are an evolutionarily conserved defense molecule, it is not surprising that bacteria have also co-evolved resistance mechanisms.

Conversely, inducible (adaptive) resistance refers to mechanisms that are induced in response to host defense peptides. Central to inducible resistance mechanisms are the two-component sensory systems (TCS). These systems induce diverse adaptive responses and provide pathogens with increased survival capabilities (Yount 2005). The physiological relevance of such defensive modifications is demonstrated in isolates of *P. aeruginosa* from cystic fibrosis (CF) patients. These isolates possess adaptive resistance responses such as Lipid A modifications and N4-aminoarabinose modified Lipid A changes, which have been presumed to increase the stability of the outer membrane (Ernst 1999). Structural changes such as these would be presumed to decrease the outer membrane permeation by HDPs. For example, the pre-exposure of wild-type *Pseudomonas* to sublethal concentrations of HDPs has been correlated with increased resistance to cationic HDPs. It has been speculated that sublethal HDP concentrations would be reflective of HDP concentrations found within the lungs of CF patients (McPhee 2003). Induction of HDP resistance in *P. aeruginosa* can be attained with multiple passage of the bacterium in sub-lethal concentrations of HDP (Zhang 2005). However, it must be appreciated that HDP resistance was modest, with only a 2- to 4-fold increases (Nizet 2006). Conversely, resistance to the aminoglycoside gentamicin under the same conditions induced resistance by 190-fold (Steinberg 1997).

Although rare, HDP resistance has been observed in nature in microbial species such as *Staphylococci, Streptococci and Salmonellae* (Peschel 2002, Ernst 2001, Nizet 2005). It has been demonstrated that such species possess genes related directly to HDP resistance (Weidenmaier 2003, Miller 2005). That HDPs have remained as an integral component of the innate immune system questions the concerns regarding HDP resistance within bacteria. Several peptides such as polymyxin B, gramicidin S, and nisin have been used in over-the-counter products without significant impact on the development of resistant strains. Much of the success of HDPs in combating pathogens is due to the metabolic cost of constitutive resistance as mutations of the microbial structures targeted by HDPs are not well tolerated by microbes (Peschel 2006).

Although HDP resistance has remained rare within microbes, it must be appreciated that the therapeutic administrations of these peptides may provoke resistance phenotypes. Indeed, the introduction of antibiotics as novel microbial eradicators was met almost instantaneously with bacteria that had manifested various forms of resistance (Tenover 2006). That noted bacteriologist and immunologist Sir Almroth Wright warned that the use of antibiotics would perpetuate the development of antibiotic resistance suggests that precautions should be taken to minimize similar resistance against HDPs.

## 1.10 The PhoPQ Two-Component System of Salmonella

Two-component systems (TCS) are amongst the primary mechanisms by which microbes assess the surrounding environment (Bishop 2006). These systems have been demonstrated to be integral for microbial virulence amongst a broad-range of bacteria,

including *Salmonella, Pseudomonas*, *Shigella, Yersinia*, and others (Groisman 2001). Typically, a TCS consists of a membrane-bound sensor histidine kinase and a cytoplasmic response regulator. Upon stimulation by a specific environmental signal, the sensor kinase autophosphorylates from ATP at a conserved histidine kinase. Following this, the sensor kinase then phosphorylates the cytoplasmic response regulator on a conserved aspartic acid residue within the 'receiver domain'.

The PhoPQ two-component system, found across a wide-range of Gram-negative bacteria, has been identified as the master regulator of virulence within many pathogenic bacteria (**Figure 1.9**) (Groisman 2001). The expression of more than 100 genes are governed by this system and approximately 1% of the open reading frames encoded within the *Salmonella* genome are directly regulated by PhoP (Groisman 2006, Groisman 2001). Although PhoPQ was initially identified as the primary controller of the expression of a non-specific acid phosphatase, hence the *pho* designation, the PhoPQ system has since been implicated in governance of virulence characteristics, adaptation to $Mg^{2+}$-limiting conditions, and regulation of numerous cellular activities (Groisman 2001). The PhoPQ system regulates the expression of a plethora of genes indispensable for survival within the phagosome. As a testament to the role of PhoPQ in *Salmonella* pathogenesis, the PhoPQ system is activated within the acidic environment of the phagosome (Alpache Arandra 1992). It has been demonstrated that PhoP-activated genes are maximally expressed within this cellular compartment (Bader 2003). It has also been demonstrated that *phoP*- and *phoQ*-null mutants are attenuated for virulence (Miller 1989). The PhoPQ system controls the expression of several genes required for microbial survival in $Mg^{2+}$-limiting environments (Groisman 2001). The survival of Salmonella within such environments requires the expression of $Mg^{2+}$ transporters. This

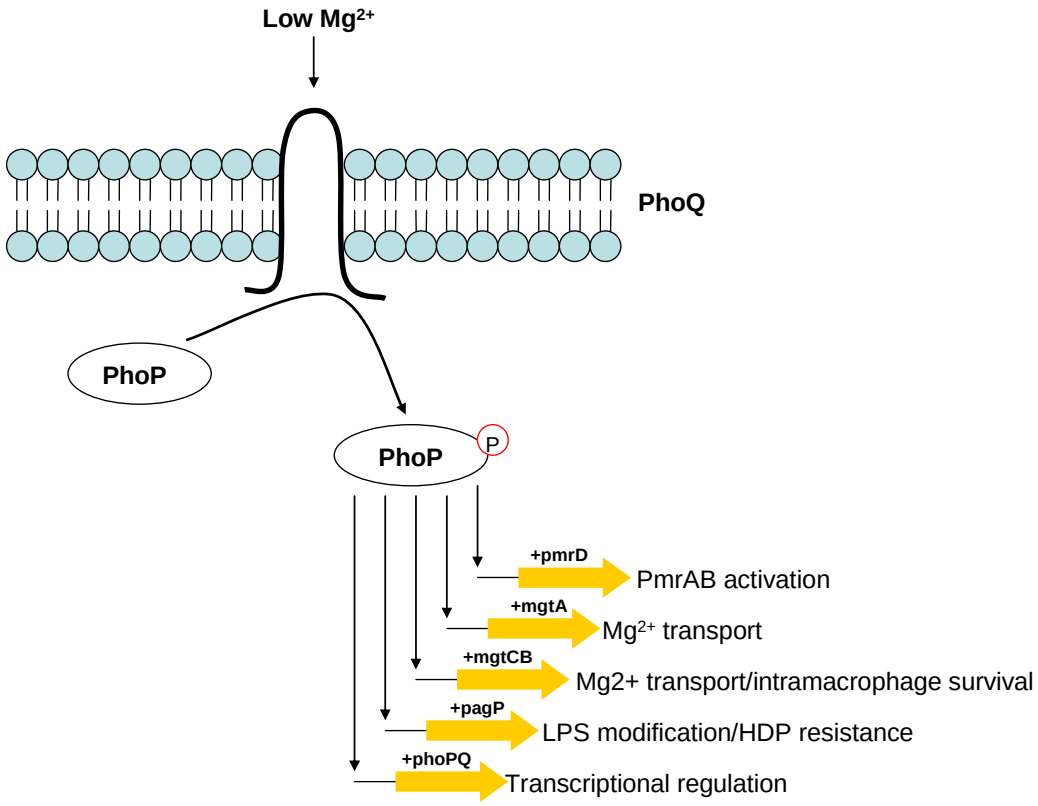



**Figure 1.9: The PhoPQ system of *Salmonella.*** Activation of the PhoPQ system within magnesium-limiting (micromolar concentrations) environments results in the induction of PhoP-regulated genes that are necessary for adaptation responses and microbial survival. Adapted from Groisman 2001.

is controlled by the *mgtA* and *mgtB* genes (Groisman 2001). Highlighting the importance of $Mg^{2+}$ transporters for microbial survival, *mgtA* and *mgtCB* null mutants reach plateau phase growth earlier than their wild-type counterparts in low $Mg^{2+}$ environments (Soncini 1996). PhoPQ also promotes the expression of genes that promote cell envelope remodeling. For example, the PhoP-regulated PagP is an outer membrane protein responsible for incorporation of palmitate into the lipid A moiety of LPS and is required for bacterial virulence (Groisman 2001). *pagP* mutants exhibit hypersensitivity to the synthetic alpha-helical peptide C18G (Guo 1998). PagL is also regulated by PhoPQ and is responsible for lipid A deacylation (Guo 1998). In contrast, PgtE, an outer membrane protease directly regulated by PhoP, is not directly involved in outer membrane remodeling. Rather, PgtE is involved in HDP inactivation by proteolytic cleavage (Sieprawska-Lupa 2004, Guina 2000). PhoPQ is also involved in the repression of genes associated with invasion phenotypes. The transcription of HilA, the primary regulator of the type III secretion apparatus and effector proteins, is repressed following activation of PhoPQ and represents a critical point of pathogen survival following invasion of host cells (Bajaj 1996). The direct and indirect expression of several transcription factors required for virulence, including SsrB (Bijlsma 2005), SlyA (Norte 2003, Shi 2004), PmrA (Kox 2000), and RpoS (Groisman 2006) are also regulated by PhoPQ.

In addition, PhoPQ also controls other two-component regulatory systems at transcriptional, post-transcriptional and post-translational levels (Bishop 2006). For example, the activation of the PhoPQ system induces transcription of *pmrD*. PmrD regulates a second two-component system, PmrAB, by preservation of the phosphorylation state of the response regulator PmrA (Kato 2004). The PmrAB-

regulated *ugd* gene and the seven-gene operon *pbgPE*, a set of PhoP-regulated genes required for growth in low $Mg^{2+}$ solid media, mediate the synthesis and incorporation of 4-aminoarabinose in the lipid A portion of LPS leading to a net reduction in the negative charge found on the bacterial membrane (Gunn 1998). It has also been recently demonstrated that PhoPQ also controls the expression of the TCS SsrBA, which regulates the transcription of the type III secretion system (Bijlsma 2005).

The TCS signaling cascade relies on an environmental signal to activate the sensor histidine kinase. *In vitro* analyses indicated that the PhoQ sensor kinase protein responded to the periplasmic levels of divalent cations such as $Mg^{2+}$ and $Ca^{2+}$ (Garcia Vescovi 1996). As PhoPQ was demonstrated to be activated *in vivo* within acidified macrophage phagosomes, the general hypothesis was formed that phagosomal $Mg^{2+}$ concentrations act as the primary environmental cue for the PhoPQ system. It has been generally accepted that low (micromolar) $Mg^{2+}$ concentrations induce the virulence-associated phenotypic changes found following phagocytosis of bacteria by activation of PhoPQ whereas high (millimolar) $Mg^{2+}$ concentrations are PhoPQ repressive (Garcia Vescovi 1996, Kato 1999, Soncini 1996). This hypothesis has recently been challenged with the demonstration that *in vivo* divalent cation concentrations are of millimolar concentrations following acidification of the phagosome and would therefore be PhoPQ-repressive (Alpuche Aranda 1992, Christensen 2002, Martin-Orozco 2006). As such, the potential for host-derived biomolecules naturally found to co-localize with bacteria in the phagosome have been investigated as prospective PhoPQ ligands. Recently, Bader has demonstrated that a central component of the innate immune system, host defense peptides, can activate PhoQ (Bader 2005)

## 1.11 PhoPQ and Host Defense Peptides

As many of the phenotypic changes induced following PhoPQ activation involve host defense peptide resistance characteristics, it has been postulated that HDPs may serve as an endogenous ligand for PhoQ (Bader 2003). Indeed, the exposure of *S. typhimurium* to sublethal concentrations of cationic antimicrobial peptides results in activation PhoPQ regulon and HDP resistance phenotypes (Bader 2003).

Employing a novel PhoPQ reporter system, the exposure of *Salmonella* to varying concentrations of HDPs (under repressing $Mg^{2+}$ concentrations) results in the direct activation of PhoQ (**Figure 1.10**) (Bader 2005). Supporting the role of HDPs as PhoQ ligands, the activation of PhoQ by HDPs was similar to that found during exposure to activating $Mg^{2+}$ concentrations (Bader 2005). HDP-mediated activation of PhoQ was also demonstrated to species-specific. A *Salmonella* mutant expressing a chimeric PhoQ in which the periplasmic domain of the sensor was replaced with that from *P. aeruginosa* failed to respond to antimicrobial peptides (Bader 2005). The chimeric receptor demonstrated wild-type responsiveness to environmental $Mg^{2+}$. This suggests that the ability of HDPs to act as endogenous ligands may have evolved in species expected to encounter host defense peptides in their respective physiological niches (Bader 2005). It was also postulated that an acidic periplasmic patch of the receptor represents the binding domain for HDPs. Bader confirmed this region as the endogenous $Mg^{2+}$ binding site by way of the crystal structure of the PhoQ periplasmic sensor domain, recapitulating the argument that this region also could act as a potential binding domain for antimicrobial peptides (Cho 2006). The authors confirmed that HDPs could bind to this region of the sensor domain and displace $Mg^{2+}$. The authors hypothesize that $Mg^{2+}$ serves to bridge the acidic periplasmic domain of PhoQ to the

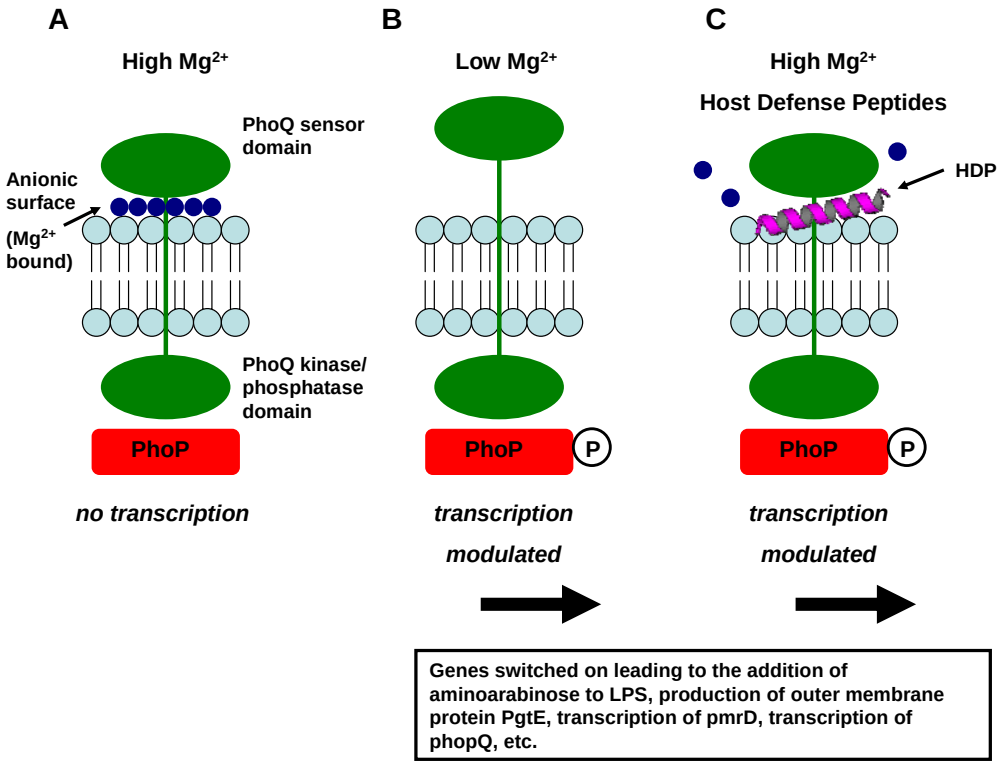



**Figure 1.10: Activation of Salmonella PhoPQ by HDPs.** Adapted from Hancock 2005. **A.** In the presence of millimolar $Mg^{2+}$ (blue circles) or other divalent cations (and the absence of HDPs), the divalent cations form a bridge between the negatively charged membrane outer leaflet and the anionic surface of the PhoQ binding domain. **B.** If micromolar concentrations of $Mg^{2+}$ are encountered the PhoQ protein undergoes a conformational change resulting in increased kinase activity of its cytoplasmic domain. This results in PhoP phosphorylation and the modulation of transcription of the *phoPQ* operon. **C.** Host defense peptides enter the bacterial outer membrane and bind to the anionic patch of the PhoQ binding domain. This results in $Mg^{2+}$ displacement and thus the activation of PhoQ (as in B). Adapted from Hancock 2005.

electronegative bacterial cell membrane. As the divalent cations shield the repulsive forces between the receptor and the membrane, the displacement by HDPs would be predicted to result in a significant repulsive force between the two moieties. Thus, it is postulated that this repulsion leads to a conformational change in PhoQ, resulting in activation (Cho 2006). This hypothesis was corroborated by the authors through mutational analysis of the highly acidic region of the PhoQ periplasmic sensor domain.

Although it has been demonstrated that PhoQ is directly activated by host defense peptides, this observation has been met with skepticism. That these systems, and in particular PhoPQ, are found in both pathogenic and non-pathogenic bacterial species calls into question the applicability of such a host defense molecule acting as an environmental signal for activation. It has been argued that commensal organisms that contain PhoPQ, such as *Escherichia coli* K-12, would not be found within tissues that harbor HDPs thus reducing the likelihood that such a ligand would be physiologically relevant in this situation (Groisman 2006). It has also been demonstrated that although micromolar $Mg^{2+}$ modulates the expression of the entire PhoP regulon, the antimicrobial peptide polymyxin B led to activation of only a subset of PhoP-regulated genes (Bader 2003). However, it has been argued that this is not unexpected as the pathogen would fine-tune the breadth of systems activated by PhoPQ depending on the stimulus, low cation concentration of HDPs (Bishop 2006).

That antimicrobial peptides do not target chiral moieties within the bacterial membrane has also questioned the ligand-potential of these host defense molecules. Indeed, the antimicrobial activity of HDPs appears to involve the direct perturbation of the bacterial cell membrane, a system that lack inherent chirality. It has also been demonstrated that the D-enantiomers of antimicrobial peptides exert similar bactericidal

activities of their natural counterparts, as well as being active across highly divergent species, reducing the potential for such peptides to be involved in classic "receptor-ligand" interactions (Chen 2006). It should be mentioned, however, that the recent demonstration of a binding domain on the sensor kinase PhoQ rather than a highly specific binding site per se would argue that the specificity of classical receptor-ligand relationships is deviated in PhoQ; a binding domain would allow for a more dynamic relationship between the sensor kinase and a potential ligand (Cho 2006).

That convincing arguments both for and against the physiological relevance of host defense peptides as ligands for the PhoPQ system reiterates the need to investigate further the potential of HDPs to initiate bacterial defensive responses and potentially the development of strains resistant to a key component of the host innate immune system arsenal.

## 1.12 Aims and Objectives

The primary objective of this research is the analysis of sensory systems from prokaryotic and eukaryotic organisms that are critical to the host-pathogen responses of each organism. From the perspective of the eukaryotic host, the Toll-like receptor system, and in particular TLR9, is examined as the biochemical characterization of the interaction between the receptor and ligand has remained inconclusive. Sensory systems within prokaryotes, and specifically *Salmonella typhimurium*, are represented by the PhoPQ two-component sensory system. As there has been a lack of consensus regarding the natural ligand of PhoPQ, host defense peptides have been recently demonstrated to activate PhoPQ (Bader 2003, Bader 2005). A set of previously characterized HDP

derivatives will be examined for their ability to either activate or evade the PhoPQ system. Retro-inverso modification of HDPs as a mechanism for increasing therapeutic stability will also be examined for the conservation of antimicrobial and immunomodulatory activities of the natural peptide.

# 2.0 Materials and Methods

## 2.1 Chemicals and Enzymes

Lists of all specialty enzymes and chemicals used in these experiments are provided in Table 2.1 and Table 2.2.

## 2.2 Bacterial Strains

*Salmonella typhimurium* is selected as the model pathogen because the majority of investigations of the PhoPQ two-component system, as well as the initial characterization of the ability of HDPs to serve as PhoQ ligands, have been performed in this bacterium. As both an enteric and intracellular pathogen, *S. typhimurium* is physiologically relevant for investigations of the host defense peptide bactenecin as the pathogen encounters bactenecin during the natural course of its infective cycle, in particular within neutrophil phagolysomes.

The strains used in this study are: American Tissue Type Culture (ATCC) 14028, a smooth virulent strain of *S. typhimurium*, PhoQ- (strain CS009 *phoQ101*:: MudJ), PhoP- (strain CS053 *phoP103*:: MudJ). To study the host defense peptide-mediated induction of the PhoP regulon, a reporter strain of *S. typhimurium* was selected (strain CS120 *phoN*::Tn*PhoA*) (Miller 1989).

The bacterial strains used for the investigation of retro-inverso modified BMAP28 were *Salmonella typhimurium* ATCC14028, *Escherichia coli* O157:H7,

**Table 2.1: List of selected chemical supplies, enzymes, and proteins**

| Chemicals | Supplier |
|---|---|
| Acetic Acid | EMD |
| Acrylamide | Sigma |
| Agarose, electrophoresis grade | Invitrogen |
| Aim V medium | Gibco, Invitrogen Corp. |
| Ammonium chloride | J.T. Baker |
| Ammonium persulfate | J.T. Baker |
| N,N-Bis(2-hydroxyethyl)-glycine | Sigma |
| Boric acid | EMD |
| 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) | Eppendorf |
| Bromophenol Blue | Sigma |
| 5-bromo-4-chloro-3-indolyl phosphate (BCIP) | Sigma |
| Chloroform:isoamyl alcohol | Amersham Biosciences |
| Coomassie-Brilliant Blue R250 | Biorad |
| Dithiothreitol (DTT) | Biorad |
| Ethidium bromide | Parmacia Biotech |
| Ethylenediamine tetraacetic acid (EDTA) | Fluka BioChemika |
| Ferrous Sulfate | J.T. Baker |
| Fetal Bovine Serum (FBS) | Gibco |
| Glycerol | Amersham Bioscience |
| Glycine | ICN Biomedicals |
| Isopropyl β-D-thiogalactopyranoside | Promega |
| Magnesium Chloride | Sigma |
| 2-mercaptoethanol | Sigma |
| Methanol | EMD |
| N,N'-Methylene-bis-acrylamide | ICN Biomedicals |
| p-nitro-blue-tetrazolium (NBT) | Sigma |

| | |
|---|---|
| p-nitro-phenol | Sigma |
| Percoll | Amersham Biosciences |
| Phenol (glass distilled) | Sigma |
| Potassium chloride | Sigma |
| Potassium phosphate monobasic | EMD |
| Potassium phosphate dibasic | EMD |
| Sodium acetate | Sigma |
| Sodium chloride | Sigma |
| Sodium dodecyl sulfate | EMD |
| Sodium hydroxide | EMD |
| Sodium phosphate monobasic | EMD |
| Sodium phosphate dibasic | EMD |
| N,N,N',N'-Tetramethylethlene-diamine (TEMED) | Pharmacia Biotech |
| 2,2,2-Trifluoroethanol (TFE) | J.T. Baker |
| Tris-[hydroxymethyl]aminomethane (ultra pure) | Biorad |
| Tween 20 | Biorad |
| Xylene cyanol | Sigma |

| **Radio Chemicals** | **Suppliers** |
|---|---|
| [$\gamma$-$^{32}$P] deoxycytosine 5'-triphosphate | Mandel |
| $H^{3}$-thymidine | Amersham Pharmacia |

| **Chromatographic Supplies** | **Suppliers** |
|---|---|
| Ni-NTA Superflow Resin | Novagen |

| **Microbiological Supplies** | **Suppliers** |
|---|---|
| Agar | BD Biosciences |
| Bacto-agar | BD Biosciences |

| | |
|---|---|
| Bacto-tryptone | BD Biosciences |
| Bacto-yeast extract | BD Biosciences |

| **Enzymes/Proteins** | **Suppliers** |
|---|---|
| Bovine Serum Albumin (BSA) | Sigma |
| T4 DNA ligase | Novagen |
| *Pfu* DNA polymerase | Stratagene |
| *Taq* DNA polymerase | New England Biolabs |
| Klenow fragment | Promega |
| PNGase F | New England Biolabs |
| RNaseH | New England Biolabs |
| Bovine TNF-α | Genetech Inc. |
| Human TNF-α | eBioscience |
| Trypsin | Boehringer Ingelheim |

| **Antibiotics** | **Suppliers** |
|---|---|
| Ampicillin | Shelton Scientific |
| Chloramphenicol | Boehringer Ingelheim |
| Kanamycin | Calbiochem |

| **Antibodies** | **Supplier** |
|---|---|
| α-TLR9 mouse mAB | EMD |
| Goat-α-mouse IgG | Kirkegarrd & Perry Laboratories |

| **Supplier** | **Addresses** |
|---|---|
| Amersham Pharmacia | Amersham Biosciences, Pittsburgh, PA, USA. |
| BD Bioscience | BD Bioscience, Mississauga, ON, Canada. |
| Biorad | Bio-Rad Laboratories Ltd., Mississauga, ON, Canada |
| Boehringer Mannheim | Boehringer Ingelheim Ltd., Laval, QC, Canada. |

| | |
|---|---|
| Calbiochem | VWR Canlab, Mississauga, ON, Canada. |
| eBioscience | eBiosciences, San Jose, CA, USA. |
| EMD Bioscience | VWR Canlab, Mississauga, ON, Canada. |
| Eppendorf | Eppendorf Canada Ltd., Mississauga, ON, Canada. |
| Fisher | Fisher Scientific, Ltd., Nepean, ON, Canada. |
| Fluka BioChemika | Sigma-Aldrich Canada Ltd., Oakville, ON, Canada. |
| Genetech Inc. | Genetech, Inc., San Francisco, CA, USA. |
| Gibco | Invitrogen Ltd., Gaithersburg, MD, USA. |
| ICN Biomedicals | ICN Biomedical Canada Ltd., Saint Laurent, PQ, Canada. |
| Invitrogen | Invitrogen Canada, Inc., Burlington, ON, Canada. |
| J.T. Baker | Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA. |
| Kirkegarrd & Perry Laboratories, Inc. | KPL, Inc., Gaithersburg, MD, USA. |
| Mandel | Mandel Scientific Co., Inc., Guelph, ON, Canada. |
| New England Biolabs | NEB Ltd., Pickering, ON, Canada. |
| Novagen | VWR Canlab, Mississauga, ON, Canada. |
| Pharmacia Biotech | Pfizer Canada, Inc., Mississauga, ON, Canada. |
| Promega | Fisher Scientific, Ltd., Nepean, ON, Canada. |
| Qiagen | Qiagen, Inc., Mississauga, ON, Canada. |
| Shelton Scientific | VWR Canlab, Mississauga, ON, Canada. |
| Sigma | Sigma-Aldrich Canada Ltd., Oakville, ON, Canada. |
| Stratagene | VWR Canlab, Mississauga, ON, Canada. |

*Serratia marcescens* ATCC 274, *Yersinia enterocolitica* O:9 E40 (pYV40), *Pseudomonas cepacia* ATCC 53795, and *Salmonella enteritidis* 27655R PT4.

## 2.3 Plasmid Vectors:

Plasmid pFLAG-CMV2 (CMV) was purchased from Sigma and pET15b was purchased from Novagen. Plasmid pBBRMCS1-4 was kindly provided by Dr. Kenneth M. Peterson, Louisiana State University.

## 2.4 Bacterial Growth Media

Media was prepared as described by Miller (Miller 1972) for Luria broth (LB) and Maniatis (Maniatis 1989) for M9 minimal media. All media was autoclaved following preparation at 125°C and 18 psi for 20 min. Liquid Luria broth (LB) media was prepared with 10 g of Bacto tryptone, 10 g NaCl, and 5 g of yeast extract in one liter of distilled water. Salt-free LB media was made as above with the exception that NaCl was not added. M9-minimal media was prepared as follows: 12.8 g of $Na_2HPO_4$, 3.0 g of $KH_2PO_4$ and 1.0 g of $NH_4Cl$ were combined in 950 ml of distilled water. The pH of the solution was adjusted to 7.4 with NaOH and the media was autoclaved for 20 min at 125°C and 18 psi. Following cooling, the following were dissolved in 50 mL of distilled water and cold filtered into the media: 2.0 g glucose, 0.01 g Thiamine, and 0.01 g $FeSO_4$-$7H_2O$.

## 2.5 Hazardous Materials

### 2.5.1 Ethidium Bromide

Ethidium bromide (EtBr) is a potent mutagen following inhalation, swallowing, or upon contact with skin. Latex gloves were used in all cases to ensure that no contact with open skin could occur. Where EtBr was used in agarose gels, EtBr (10 mg/mL) was added prior to polymerizing of the agarose and gels were disposed of in a designated EtBr waste container (Quillardet 1988).

### 2.5.2 Phenol

Phenol is toxic upon inhalation, swallowing, or contact with exposed skin. Phenol was always used in well-ventilated areas and contact minimized by use of latex gloves. All samples containing phenol were collected and discarded as chemical waste.

### 2.5.3 Chloroform

Chloroform is toxic upon inhalation and may result in kidney and/or liver damage. All work utilizing chloroform was conducted in a fumehood and contact with skin was minimized by use of latex gloves. Samples containing chloroform were collected and treated as chemical waste.

### 2.5.4 Acrylamide

In the unpolymerized form, acrylamide is a potent neurotoxin and potential carcinogen upon inhalation, swallowing, or contact with exposed skin. Acrylamide

solutions (40%) were prepared by dissolving 38.0 g of acrylamide and 2.0 g of bis-acrylamide in 100 mL of deionized water with vigorous mixing and heating. Preparation of acrylamide solution was conducted in a well ventilated area with a facial filtering mask and latex gloves. All acrylamide solutions were polymerized prior to disposal as regular waste.

## 2.6 SDS-PAGE Analysis

SDS-PAGE gels were prepared using the Laemmli method (Laemmli 1970), and run using a discontinuous buffer system as described by Sambrook (Sambrook 1989). Following electrophoresis, gels were stained for 1 hour in Coomassie Brilliant Blue (45% methanol, 10% glacial acetic acid, 0.25% w/v Coomassie Brilliant Blue R250) and destained in destaining solution (7.5% methanol, 10% glacial acetic acid) overnight.

## 2.7 'Mini-prep' Isolation of Plasmid DNA

DNA plasmid isolation was performed through the mini-prep procedure as previously described (Birnboim 1979, Maniatis 1982). *E. coli* tuner DE3 pLysS cells transformed with the appropriate plasmid were grown overnight at 37°C with constant shaking in LB media supplemented with ampicillin (50 µg/mL) and chloramphenicol (34 µg/mL). Cells were isolated from the cultured media by centrifugation (14,000 x g for 10 min) of a 1.5 mL aliquot in a microfuge tube. The supernatant was removed by suction and the pellet was resuspended in 100 µl of Solution I (50 mM glucose, 25 mM Tris-HCl buffer pH 8.0 and 10 mM EDTA) and placed on ice for 1 minute followed by

addition of 200 µl freshly prepared Solution II (0.2 N NaOH, 1% SDS). The mirofuge tube was then gently swirled by hand and stored on ice for 5 min followed by the addition of 150 µl of Solution III (5 M sodium acetate, pH 5.2) and subsequent vigorous vortexing. The tube was then incubated on ice for 5 min followed by centrifugation for 5 min at 12,000 x g and subsequent transfer of the resultant supernatant to a fresh microfuge tube. Equal parts (250 µL) of phenol and chloroform:isoamyl alcohol (1:24) were added and the solution was mixed by inversion and centrifuged for 2 min a 14,000 x g. The upper aqueous layer was transferred to a new Eppendorf tube and the plasmid DNA was precipitated with the addition of 2 volumes (1 mL) of cold 95% ethanol, followed by an incubation of ice for at least 10 min and subsequent centrifugation for 15 min at 14,000 x g at 4°C. The supernatant was gently removed with care to not disrupt the pellet, and the pellet was then washed with cold 70% ethanol and centrifuged at 14,000 x g for 15 min at 4°C. The supernatant was again removed gently and the pellet was air dried. Finally, the dried pellet was resuspended in 30 µL of TE buffer (10 mM Tris-HCl buffer pH 7.5, 1 mM EDTA) with 1 µl of RNase (10 mg/mL solution, boiled for 10 min) and stored at -20 C.

## 2.8 *In vitro* and *in vivo* Analysis of the Interactions of Single-Stranded ODNs and Double-Stranded DNA Plasmids with TLR9

### 2.8.1 Cloning of TLR9

With some species-specific variations, TLR9 is a protein of approximately 1030 amino acids; the N-terminal 750 residues contain 25 LRR motifs, residues 750-810 an

extracellular C-terminal motif, residues 810-825 represent the transmembrane region and residues 825-1030 the intracellular TIR domain. The entire ectodomain, residues 1-815, were selected to represent the TLR9 ligand-binding domain, TLR9(LBD), as the specific ligand-binding region of the receptor has not yet been identified.

For expression of the TLR9(LBD) protein, a eukaryotic expression system employing the bovine hsp70A gene promoter was utilized. This system directs the heat-regulated synthesis of proteins in transfected bovine kidney epithelial cells and has proven highly effective for the overexpression of other glycosylated proteins (Kowalski 1993).

DNA for the TLR9 ectodomain was PCR amplified, with the addition of a six residue histidine tag at the carboxy-terminus, using a plasmid containing the human TLR9 ORF as a template (kindly provided by Grayson Lipford). The resulting PCR product was cloned downstream of a bovine hsp70A gene promoter and the expression cassette was stably introduced into MBDK cells by Lentiviral delivery (Rouas 2002).

### 2.8.2 TLR9 Overexpression

The eukaryotic expression system utilized in this analysis results in the secretion of recombinant proteins into the culture media, greatly facilitating subsequent purification steps and represents an inherent advantage of this system. Due to the non-destructive cyclic nature of this expression system, cultures could be split during ongoing heat-shock cycling yielding relatively low quantities of protein (approximately 10 μg of protein per liter of culture media). The large-scale production of protein due to the ongoing cycling steps allow for sufficient quantities of protein to be generated.

Sufficient quantities of the protein could be produced from 10 rounds of temperature shifting, six hours at 39°C followed by 18 hours at 37°C, of forty T150 flasks. The secreted protein was further purified using commercially available Nickel-columns (Novagen). Purified TLR9(LBD) was dialyzed against 20 mM phosphate buffered saline (pH 7.2), 100 mM NaCl, 0.5 mM β-mercaptoethanol.

### 2.8.3 Deglycosylation of TLR9(LBD)

Purified TLR9(LBD) was treated with peptide N-glycosidase F (PNGase F) as follows: 20 μg of TLR9(LBD) was denatured in 5% SDS and 0.4 M DTT for 10 minutes at 100 °C. Following this, reaction buffer (0.5 M sodium phosphate, pH 7.5) and 10% NP-40 were added at 1/10 reaction volume with 500 units of PNGase F. The deglycosylation was allowed to proceed at 37 °C for 1 hour.

### 2.8.4 Agarose Electrophoretic Mobility Shift Assays

Unless otherwise specified, DNA binding assays were performed with the incubation of 8 μg of TLR9(LBD) [20 mM phosphate buffered saline (pH 7.2), 100 mM NaCl, 0.5 mM β-mercaptoethanol] with 1.4 μg of DNA (pFLAG-CMV-2 expression vector (Sigma)) in the presence of 10 mM $MgCl_2$. Reactions were buffered with 100 mM Na acetate (pH 5.2) unless otherwise specified. The final volume of each reaction was 50 μL. Reactions were incubated at 37°C for 10 min and stopped with the addition of 10 μL agarose loading solution (30% glycerol) and immediately electrophoresed

through a 0.8% agarose gel at 95 V for 1 hour. DNA visualization was through ethidium bromide staining.

### 2.8.5 Agarose Electrophoretic Supershift Assays

Supershift assays were performed by the same methodology as described for the mobility shift assays with the exceptions that the TLR9(LBD) was dialyzed against (50 mM phosphate buffered saline (pH 7.2), 100 mM NaCl) and the reactions were performed at pH 7.2 (50 mM Tris). The TLR9 monoclonal antibody was purchased from Oncogene Research Products. The reaction mixture was incubated for 1 hour at 37°C prior to electrophoresis.

### 2.8.6 Freeze-Squeeze Method for Protein Extraction from Agarose

Following agarose electrophoresis, bands corresponding to the nucleoprotein complexes were excised and placed in microfuge tubes. The tubes were frozen at -80°C overnight. Samples were thawed at room temperature and centrifuged at 14,000 rpm for 10 minutes. The supernatant was extracted for further analysis.

### 2.8.7 Western Blot Analysis

Proteins were resolved through a 7.5% SDS-PAGE gel. Following electrophoresis, the proteins were transblotted onto polyvinylidene difluoride membranes (PolyScreen; Perkin Elmer Life Sciences) for 1 hour at 0.3 Amps. Membranes were blocked with 3% BSA in TBST (20 mM Tris pH 7.6, 0.14 M NaCl.,

0.02% (v/v) Tween 20) for 1 hour at room temperature and then exposed overnight to the primary antibody at 4°C. Following the primary incubation, membranes were rinsed in TBST and incubated with the secondary antibody for 1 hour at room temperature in TBST with 3% molecular grade fat-free skim milk powder. Membranes were subsequently washed with TBST and developed in AP buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) supplemented with 15mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 30 mg/ml p-nitro-blue-tetrazolium (NBT). The monoclonal antibody to human TLR9 (Oncogene Research Products (EMD Biosciences)), α−hTLR9 was used at a dilution of at 4 μL/mL. The secondary antibody [goat-α−mouse IgG (H+L) (Kirkegarrd & Perry Laboratories, Inc.)] was used at 0.5 μL/mL to probe for TLR9-antibody complexes.

### 2.8.8 Acrylamide Electrophoretic Mobility Shift Assays

ODNs were labeled with $^{32}P$ using [γ-$^{32}P$]-dCTP (3000 Ci/mmole) and Klenow fragment of DNA polymerase I as follows: 2 μl of 10x Klenow fragment of DNA polymerase I buffer (500 mM Tris–HCl (pH 8.0), 50 mM $MgCl_2$ and 10 mM DTT), 2 μl of 20 μM duplexes (mouse and human CpG motif sense ODNs and the respective antisense ODNs) were annealed before use in equimolar amounts and 16 μl of $H_2O$ were added to 120 μCi [γ-$^{32}P$]-dCTP. After 15 min at room temperature, 1 μl of 10 U/μl Klenow fragment of DNA polymerase I (Promega) was added. After 15 min, 2 μl of 100 mM dNTP was added, and after 15 min incubation, the reactions were stopped with 1.5

μl of 0.5 M EDTA. The reaction mixtures were purified by MicroSpin G-25 columns (Amersham Pharmacia Biotech) to remove free [$\gamma$-$^{32}$P]-dCTP.

Binding reactions containing 0.2 ng of double-stranded labeled probe, 8 μg TLR9(LBD), 10 mM Tris (pH 7.8), 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT and 5% glycerol were incubated at 37°C for 20 minutes. Non-denaturing polyacrylamide gels (5%) containing 8.9 mM Tris (pH 8.0), 8.9 mM Boric Acid, 1 mM EDTA were pre-run at 5 v/cm for 30 minutes at 20°C prior to loading of the binding reactions. Samples were loaded onto acrylamide gels with the addition of 5 μl non-denaturing loading dye (50% v/v glycerol, 0.005% w/v bromophenol blue, 0.005% xylene cyanol) and electrophoresed for 2 hours at 10 V/cm. Gels were dried for 1 hour at 80 °C and exposed to a Kodak Storage Phosphor screen GP. Storage Phosphor Screens were scanned with a Bio-Rad Molecular Imager FX using Bio-Rad Quantity One Software.

### 2.8.9 Cell Proliferation Assays

The isolation and culture of bovine peripheral blood mononuclear cells (PBMC) was as previously described (Mena 2003). Briefly, PBMC were isolated from 3, mature (> one year old), male and female cattle. Lymphocyte proliferative responses for each animal were assayed by stimulating triplicate cultures (2 X $10^5$ cells/well) in a U-bottom 96-well culture plate. Cells were cultured for 72 h at 37°C in a humidified atmosphere of 5% $CO_2$ and 0.4 μCi of $H^3$-thymidine (Amersham Pharmacia, Piscataway, NJ) was added to each well. Cells were harvested using standard liquid scintillation protocols and the incorporation of $H^3$ was measured using a beta-counter (Topcount, Packard

Instrument Company, Meriden, CT). The lymphocyte proliferative response was calculated as the mean counts per minute for the triplicate cultures.

### 2.8.10 Oligodeoxynucleotides

The sequences of the ODNs used in this investigation are presented in **Table 2.2**. The PTO versions of the 2007 ODNs consist exclusively of phosphothioate linkages. PD-ODNs were purchased from Invitrogen and PTO-ODNs were kindly provided by Qiagen.

**Table 2.2: Oligodeoxynucleotides Used in this Investigation.**

| ODN Name | Sequence |
|---|---|
| Human CpG | GGATCAGCGGAGGTCGTTTTGTCGTTCTCTGTC |
| Human GpC | GGATCGACAGAGAACGACAAAACGACCTCCGCT |
| Mouse CpG | GGATCAGCGGAGGACGTTCTGACGTTCTCTGTC |
| Mouse GpC | GGATCGACAGAGAACGTCAGAACGTCCTCCGCT |
| 2007 CpG | TCGTCGTTGTCGTTTTGTCGTT |
| 2007 GpC | TGCTGCTTGTGCTTTTGTGCTT |

## 2.9 Investigation of HDPs for Antimicrobial Activity and Bacterial Defensive Response Stimulation in *Salmonella typhimurium*

### 2.9.1 Cloning and Expression of PhoPQ from *S. typhimurium*

Plasmid pBBR1MCS-4-*phoPQ* was constructed through PCR amplification of the chromosomal *phoPQ* operon, including promoter region, from wild-type *S. typhimurium* with primers A AAAGTCGGGCCAGTTAAG and B CGCCGGCAAATTATATCG using *S. typhimurium* chromosomal DNA as template. The resulting PCR product was cloned directly into the EcoRV site of pBBR1MCS-4 to generate plasmid pBBR1MCS-4-*phoPQ* (Kovach 1995).

### 2.9.2 Functional Reconstitution of PhoP$^-$ and PhoQ$^-$

The vector containing the Salmonella PhoPQ operon, pBBR1MCS-4-PhoPQ, was transformed into Salmonella PhoP$^-$ and PhoQ$^-$ strains to the creation of PhoP$^{recon}$ and PhoQ$^{recon}$, respectively.

### 2.9.3 Host Defense Peptide Selection

The HDP bactenecin, also known as bovine dodecapeptide, is a small cathelicidin discovered in bovine neutrophils (Romeo 1988). At twelve amino acids in length, bactenecin is one of the smallest active HDPs. Its small size and extensive characterization make it an ideal model peptide for examination of the consequences of amino acid substitutions on antimicrobial efficiency. While the natural peptide is stabilized by an internal disulfide bridge, a linear variant, Bac2A, (RLARIVVIRVAR-$NH_2$), shows a similar activity against Gram-negative bacteria and higher activity against Gram-positive bacteria (Wu 1999). Others have reported on the antimicrobial properties of a complete substitution library of Bac2A against *P. aeruginosa,* as well as

a partial screen against *S. typhimurium* (Hilpert 2005). A series of Bac2A derivatives was selected based on the reported antimicrobial efficiencies against *Salmonella*, which have improved, maintained or compromised antimicrobial activity as a consequence of the sequence manipulation. The nomenclature of the peptide derivatives is maintained from the original publication (Hilpert 2005). A negative control (NC) peptide of unrelated sequence, GATPEDLNQKLS-$NH_2$, is also included (Hilpert 2005).

### 2.9.4 Peptide Synthesis

Peptides were chemically synthesized by Chemistry Services at VIDO on a Pioneer solid-phase peptide synthesizer (PerSeptive Biosystems, Foster City, CA) using Fmoc (9-fluorenylmethoxy carbonyl) chemistry. The peptide chains were synthesized from the carboxyl terminus to the amino terminus onto [5-(4-Fmoc-aminomethyl-3,5-dimethyloxyphenoxy) valeric acid]-polyethylene glycol-polystyrene (PAL-PEG-PS) resin. Both Fmoc-protecting groups at the amino terminus were deprotected with piperidine. The peptides were cleaved from the resin with concurrent deprotection of the side chain-protecting groups by treating the resin-bound peptide with trifluoroacetic acid (TFA) (9.3 parts) in the presence of scavengers (anisole-ethyl-methyl sulfide-1,2-ethanedithiol [3:3:1]), for 7 h. The crude peptides were filtered from the resin, and the TFA was evaporated. Diethyl ether was added to the residues to precipitate the crude peptide. The peptides were isolated and purified by high-performance liquid chromatography (HPLC) on Vydac protein $C_4$ columns (1.0 by 25 cm) eluting with a linear gradient of 35% buffer A ($H_2O$-0.1% TFA)-90% buffer B (acetonitrile-$H_2O$ [90/10]-0.01% TFA) for 40 min at a flow rate of 3 ml/minute. The purity and molecular

weight of the respective peptides were confirmed by matrix-assisted laser desorption ionization (MALDI)-time of flight mass spectrometry on a PE Biosystems Voyager system 4068 (National Research Council, Plant Biotechnology Institute, Saskatoon, Canada) and by amino acid analysis.

### 2.9.5 Determination of Minimal Inhibitory Concentrations (MIC)

The minimum inhibitory concentrations (MICs) of the peptides were determined using a modified broth micro-dilution method (Wu 1999). Briefly, overnight cultures of bacteria were diluted to a concentration of 5 X $10^5$ bacteria per mL in salt-free LB medium. Assays were performed in sterile 96-well, round-bottom polypropylene microtitre plates with a 45ul aliquot of the appropriate bacterial strain in the presence of varying concentrations of the HDP (15ul of peptide in each well). Peptide stocks were made in $dH_2O$ at a concentration of 2 mg/mL and stored at -20°C. Appropriate dilutions of the peptides were made fresh prior to each MIC trial in 0.2% BSA, 0.01% acetic acid as previously described (Wu 1999) and added to the culture following addition of the bacterial inoculum in the appropriate microtiter plate well position. The plates were incubated at 37°C for 16 h, and well solutions spotted onto LB-agar plates. The MIC was taken as the concentration at which no growth was observed. Each MIC assay was performed in triplicate from three different bacterial growths. The MICs reported represent the averages of these results.

### 2.9.6 Activation of Adaptive Responses by Low Magnesium and Host Defense Peptides

The extent to which host defense peptides can activate adaptive responses, as determined by increased MIC values, was determined through a modification of the standard MIC assays. Bacterial cultures were pre-incubated in a 96 well plate for 4 h in the presence of increasing sub-lethal concentrations of the HDP under investigation (1 μg/ml of HDP administered at time 0, 1 μg/ml addition at 1 h, 2 μg/ml addition at 2 h, and a final addition of 4 μg/ml at 3 h). Following this adaptation period, bacteria were examined through standard MIC assays to quantify shifts in HDP susceptibility. For induction of adaptive responses by low magnesium conditions, bacteria were grown overnight in N-minimal media with the inclusion of either 10 μM or 10 mM $MgCl_2$.

### 2.9.7 PhoPQ Reporter Assays

*S. typhimurium* strain CS120 (kindly provided by Dr. Samuel I. Miller, University of Washington), expressing a PhoP-regulated fusion between PhoP-dependent acid phosphatase (PhoN) and *E. coli* alkaline phosphatase (PhoA), was utilized for analysis of PhoPQ activation. The activity of this fusion protein was measured in growth media containing varying concentrations of the peptides and/or magnesium. The assay conditions utilized are based on previously described protocols (Brickman 1975).

### 2.9.8 Alkaline Phosphatase Reactions

For quantification of alkaline phosphatase induction, *S. typhimurium* strain CS120 was incubated with the appropriate peptide derivative or magnesium for 3 h at the varying concentrations in $Mg^{2+}$-free N-minimal media. Following the incubations, 250 µl of culture from each sample was brought to 2.4 ml with 50 mM Tris buffer (50 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$) and incubated for 3 min at 37°C. 0.1 ml of 0.5% (w/v) p-Nitrophenyl-Phosphate (NPP) was added to each tube and incubated at 37°C. The reactions were monitored for a change in color and stopped by addition of 0.5 ml of 1 M Potassium Phosphate buffer (pH 8.0). The absorbance of each sample was measured at 410 nm.

### 2.9.9 Proteolytic Digests of BMAP28 and RI-BMAP28

Both the natural BMAP-28 and RI-BMAP-28 (1 mg/mL) were digested with trypsin (0.1 mg/ml) in a 50 µL reaction volume (50 mM Tris pH 7.2) at 37°C for a series of time points. For peptide proteolysis characterization, the peptides were isolated by high-performance liquid chromatography (HPLC) for comparison of peak areas relative to an undigested standard on Vydac protein $C_4$ columns (1.0 by 25 cm) eluting with a linear gradient of 35% buffer A ($H_2O$-0.1% TFA)-90% buffer B (acetonitrile-$H_2O$ [90/10]-0.01% TFA) for 30 min at a flow rate of 1 ml/minute.

### 2.9.10 Measurement of Hemolytic Activity (MHC)

Peptide samples were added to 1% bovine erythrocytes in phosphate-buffered saline and reactions were incubated at 37°C for 18 h in microtiter plates. Appropriate dilutions of the peptides were made fresh prior to each MHC trial in HDP dilution buffer (0.2% BSA, 0.01% acetic acid) and added following addition of the erythrocytes in the appropriate microtiter plate well position. Hemolysis was determined by withdrawing aliquots from the hemolysis assays, removing unlysed erythrocytes by centrifugation (800 x g) and determining which peptide concentrations cause hemoglobin release. Hemoglobin release was determined spectrophotometrically at 570 nm. 1% erythrocytes with no added peptide served as a non-lysis control. As erythrocytes were incubated in an isotonic medium, no detectable release (<1% of that released upon complete hemolysis) of hemoglobin was observed from this control during the course of the assay. 1% erethrocytes were also incubated with the peptide dilution buffer and no detectable release of hemoglobin was observed during the course of the assay.

### 2.9.11 Characterization of Secondary Structure

The mean residue molar ellipticities of peptides were determined by CD spectroscopy, using a Jasco J-720 spectropolarimeter (Jasco, Easton, MD), at 5°C under non-denaturing conditions (50 mM $KH_2PO_4/K_2HPO_4$, 100 mM KCl, pH 7), as well as in the presence of an $\alpha$-helix inducing agent, 2,2,2-trifluoroethanol (TFE) (50 mM $KH_2PO_4/K_2HPO_4$, 100 mM KCl, pH 7 buffer, 50% TFE). Peptides were diluted 10-fold from a 500 μM stock solution and loaded into a 0.02-cm fused silica cell. Ellipticity was

determined by scanning from 190 to 250 nm. Deconvolution of the CD spectra data was carried out using the program CD Spectra Deconvolution Version 2.1.

### 2.9.12 Capture ELISAs

Untreated peripheral blood mononuclear cells (PBMCs) or PBMCs treated with LPS in the presence or absence of the HDPs were centrifuged at 1000 g for 5 min followed by 10,000 g for 10 min to obtain cell-free tissue culture supernatant samples. The supernatants were subsequently aliquoted and stored at -20 C until further use. TNF-α secretion was monitored with a species-specific capture ELISA. All assays were performed in triplicate. The concentration of the cytokine was quantified by establishing a standard curve with serial dilutions of the recombinant human TNF-α (eBioscience, San Diego, CA) or bovine TNF-α (Genetech Inc. San Francisco, CA).

# 3.0 Results

## 3.1 Analysis of the TLR9-Ligand Interaction *in vitro* and *in vivo*

### 3.1.1 Expression of TLR9(LBD)

Central to the biochemical characterization presented here is the requirement for substantial quantities of TLR9 with conservation of the normal post-translational modifications (PTMs) found on the endogenous receptor. To provide for PTM patterns similar to those found within the host, a eukaryotic expression system was employed. Utilizing the bovine hsp70A gene promoter, this expression system directs the heat-shock regulated synthesis of proteins in transfected bovine kidney epithelial cells. This system has proven effective for production of other soluble glycosylated proteins (Kowalski 1993). Although protein yields per cycle are low, the system is robust and the cells can be cycled through multiple heat shock steps to attain the quantity of protein desired. An added feature of the heat-shock expression system is that expressed proteins are secreted into the surrounding media. Thus, the need for cell lysis steps following protein expression is circumvented.

The primarye objective of the eukaryotic expression system was to obtain sufficient quantities of the recombinant receptor for the biochemical characterization of TLR9 ligand binding. The characterization of TLR9 ligand binding would

employ mobility shift assays and, providing verification of the presence of the receptor, is not dependent on absolute purification of the receptor. As the ectodomain of the TLRs is responsible for ligand binding, expression of the TLR9 ligand binding domain, TLR9(LBD), would be adequate for this investigation. Following TLR9(LBD) expression, western blot analysis with a human TLR9 ectodomain monoclonal antibody confirmed a single protein species with a mass consistent with previous reports for the glycosylated TLR9 (Latz 2004). Untransfected control cells were devoid of any reactivity towards the monoclonal TLR9 antibody (**Figure 3.1a**).

To confirm that the TLR9(LBD) was glycosylated, the receptor was treated with the deglycosylase PNGase F, which catalyzes the removal of both N- and O-linked glycosylations. Western blot analysis with the human TLR9 monoclonal antibody confirmed a protein species of approximately 100 kDa, consistent with the predicted mass of the non-glycosylated polypeptide and with previous observations monitoring TLR9 deglycosylation (Latz 2004) (**Figure 3.1B**).

### 3.1.2 TLR9(LBD) Retains DNA Binding Capabilities

That the natural ligand for TLR9 is unmethylated CpG motifs suggests that the receptor may recognize these microbial signatures within different configurations of DNA. Indeed, plasmid DNA initiates innate immune responses (Krieg 1995), and the endosomal stability of plasmids allows them to serve as TLR ligands (Bennett 1985). These observations are suggestive of a physiological interaction between TLR9 and plasmids. SPR analyses by Cornelie (Cornelie 2004) and Rutz (Rutz 2004) lack consensus regarding the affinity of the receptor for double-stranded DNA. The

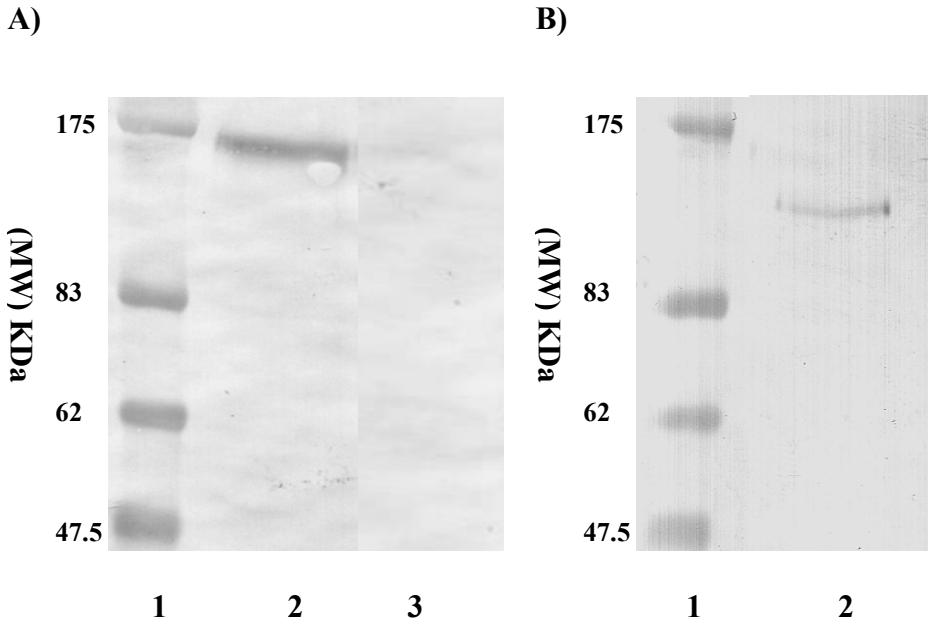



**Figure 3.1: Expression of TLR9(LBD).** Proteins were resolved through a 10% SDS-PAGE gel and subjected to Western blot analysis with a monoclonal antibody to human TLR9. **A) Confirmation of TLR9(LBD) Synthesis:** Lane 1: Molecular weight marker, Lane 2: 10 μg TLR9(LBD) and Lane 3: Media collected from untransfected cells. **B) Deglycosylation of TLR(LBD)** Carbohydrate groups were removed from TLR9(LBD) by treatment with PNGase F. Lane 1: Molecular weight marker, Lane 2: 10 μg deglycosylated TLR9(LBD). Protein sources for the molecular weight marker are as follows: **175 KDa**, MBP-β-galactosidase (*E. coli*); **83 KDa**, MBP-paramyosin (*E. coli*); **62 KDa**, MBP-CBD (*E. coli*); **47.5 KDa** Aldolase (rabbit muscle).

Investigation by Cornelie noted that the receptor bound plasmid with high affinity, whereas Rutz reported minimal binding of linear double stranded ODN molecules with internal CpG motifs (Cornelie 2004, Rutz 2004).

To address the interaction of TLR9 with plasmid DNA, a modified agarose electrophoretic mobility shift assay (AEMSA) was employed. This system offers a low-cost, highly-reproducible method for studying DNA binding. As an analytical tool, AEMSA provides information regarding nucleoprotein complex formation based on differential patterns of DNA migration in the presence and absence of DNA-binding proteins. The resolution of the nucleoprotein complexes relies largely on the stability of the nucleoprotein complex within agarose gels (Lane 1992). The stability of nucleoprotein complexes within an agarose gel are increased above that of their kinetic stabilities within free solution due to "caging effects" of the gel matrix (Lane 1992). This caging effect impedes the diffusion of dissociated components thus conserving the concentrations of protein, DNA, and nucleoprotein complex at levels equivalent to those in the original binding reaction (Fried 1981). Previous investigation of nucleoprotein complex formation between holo-TFIID and the Ea promoter lend support for the use of AEMSA in this investigation (Frontini 2002). This system has two distinct advantages in contrast to traditional investigation of DNA-protein interactions. Firstly, the increased porosity of agarose allows large nucleoprotein complexes to be resolved, a problem in traditional acrylamide EMSA. Secondly, AEMSA does not require immobilization of either the receptor or ligand. Thus, the natural dynamics of the receptor-ligand interaction are maintained.

Within agarose gels, plasmids normally resolve to two predominant species: a faster-moving supercoiled form and a slower-moving relaxed form (Oppenheim 1981). In the presence of TLR9(LBD), plasmid migration is shifted and results in the formation of two distinctly migrating species (**Figure 3.2a**). It is also of note that the faster moving unbound supercoiled plasmid band is decreased in concentration. The two novel nucleoprotein complexes formed likely correspond to different stoichiometric ratios of receptor to plasmid. These novel species are hypothesized to represent monomeric and dimeric forms of the receptor bound to a single plasmid molecule. Ligand-induced dimerization continues to emerge as a common theme within the Toll and Toll-like family (Takeuchi 2001, Gangloff 2004). It has also been reported that the formation of a monomeric Toll-Spätzle complex is a prerequisite for the formation of the active dimeric Toll complex (Gay 2006, Weber 2005).

The interaction between plasmid DNA and TLR9 is dependent upon the maintenance of a correctly folded structure. Deliberate disruption of the TLR9(LBD) protein structure prior to its addition to the reaction mixture abolishes DNA binding activity (**Figure 3.2a**). This verifies that the recombinant TLR9 is functional and that the receptor is able to bind double-stranded DNA. Notably, the recombinant receptor preferentially binds supercoiled plasmids, which may account for the discrepancies previously reported in the literature. The lack of consensus regarding the interaction between TLR9 and double-stranded DNA likely results from different representations of the ligand. The investigation by Cornelie utilized double-stranded linear ODNs which cannot adopt a supercoiled configuration (Cornelie 2004).

To verify that the differential patterns of migration result from the direct binding of plasmids by TLR9(LBD), a supershift assay was performed employing the

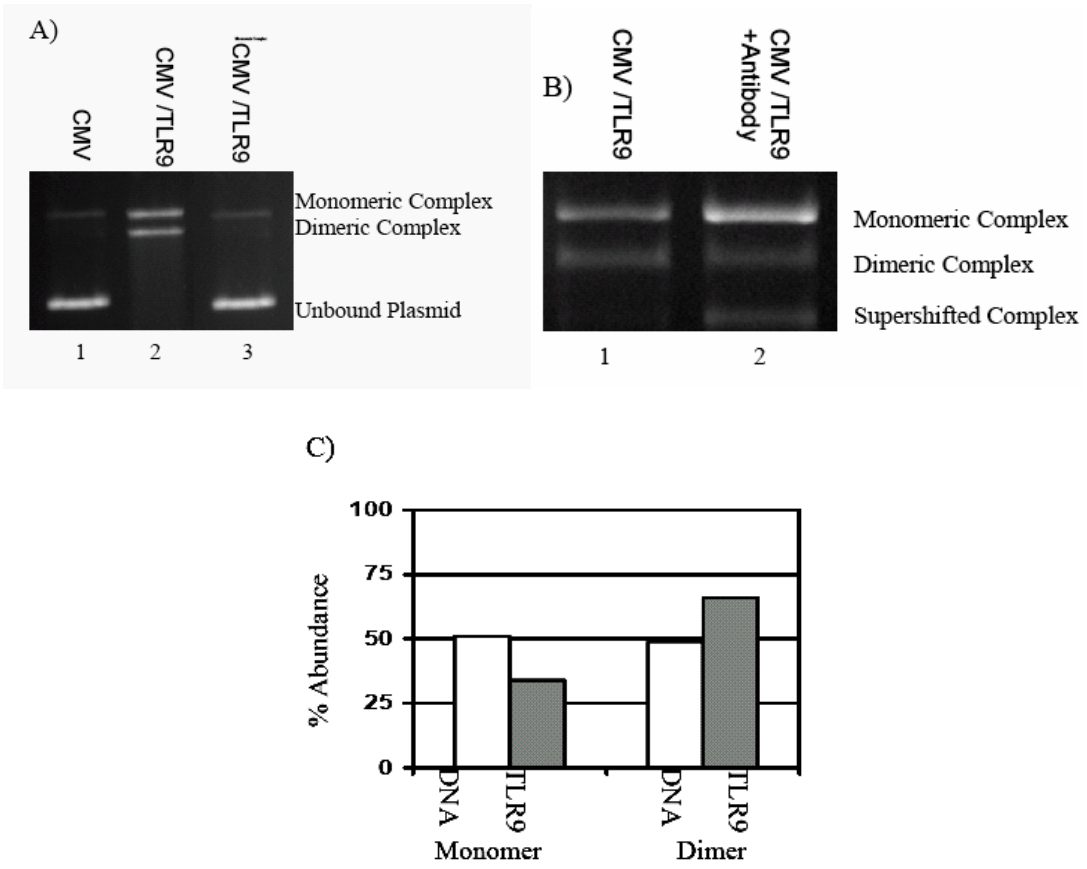



**Figure 3.2: TLR9(LDB) Binds Plasmids.** Standard reaction conditions were employed as described previously with the exception of the pH adjustment for the supershift assay. CMV refers to pFLAG-CMV-2. **A) TLR9(LBD) Binds Plasmid to the Formation of Two Distinct Nucleoprotein Complexes.** Lane 1: CMV; Lane 2: CMV and TLR9(LBD) and Lane 3: CMV and TLR9(LBD) boiled for 15 minutes prior to addition to the binding assay. Reactions were buffered at pH 5.2 **B) Supershift Binding Assays**. Lane 1: CMV and TLR9(LBD) and Lane 2: CMV and TLR9(LBD) and a monoclonal antibody specific to the ectodomain of human TLR9 (1.5 μg). For A and B CMV and TLR9(LBD) are at constant concentrations of 1.4 μg and 8 μg, respectively, and reaction mixtures were buffered at pH 7.2. **C) Confirmation of Monomeric and Dimeric Nucleoprotein Complexes.** The stoichiometry of the shifted nucleoprotein complexes was determined by comparing the relative abundances of both nucleic acid and TLR9(LBD) within uniquely migrating species hypothesized to represent monomeric and dimeric forms of the TLR9 nucleoprotein complex. Relative band intensities were calculated for both the ethidium stained agarose gel to determine relative nucleic acid content as well as for Western blots of proteins extracted from these gel slices. Results represent the averages of two trials. Densitometry analysis was performed with the AlphaEase imaging system.

monoclonal human TLR9 antibody. The incorporation of the monoclonal antibody into the nucleoprotein complex will result in a shifted pattern of migration as compared to that of the nucleoprotein complex in the absence of antibody. The addition of the monoclonal antibody induced a novel pattern of migration confirming the presence of TLR9(LBD) in the nucleoprotein complex (**Figure 3.2b**).

The stoichiometry of nucleic acid and TLR9(LBD) within the shifted nucleoprotein complexes was determined to confirm existence of monomeric and dimeric forms of the receptor. Shifted bands were excised from the gel shift assay and analyzed for plasmid and TLR9(LBD) concentrations. The concentrations of plasmid DNA in both the proposed monomeric and dimeric species were confirmed to be equivalent by quantification of band intensity using the AlphaEase imaging system. To determine the relative concentration of TLR9(LBD) present within each complex, the shifted bands were excised and proteins were extracted by the freeze-and-squeeze method. Western blot analysis with the monoclonal human TLR9 antibody confirmed that the intensity of the signal in the lane corresponding to the proposed dimeric form of the receptor is 1.95 times the intensity of signal in the lane corresponding to the monomeric form of TLR9 (**Figure 3.2c).**

### 3.1.3 pH Dependence of DNA Binding

Although limited information regarding the direct interaction between TLR9 and DNA has been reported, it has been conclusively demonstrated that the interaction between TLR9 and CpG-DNA occurs within the endosomal compartment (Ahmad-Nejad 2002). Notably, TLR9 and DNA co-localize to the endosome following

endocytosis of ODNs and TLR9-mediated responses require acidification and maturation of endosomes (Macfarlane 1998). Consistent with these results, Rutz has demonstrated that the formation of stable nucleoprotein complexes is highly pH-specific (Rutz 2004). The ability of TLR9(LBD) to bind plasmids was monitored at both acidic (5.2) and neutral (7.5) pH. TLR9(LBD) binds plasmids more efficiently under acidic conditions as demonstrated by the formation of stable nucleoprotein complexes at lower concentrations of the recombinant receptor (**Figure 3.3**). This pH dependence is in accordance with previous investigations by Rutz and the binding of double-stranded RNA by TLR3 (Rutz 2004, de Bouteiller 2005).

It is of note that complex formation between the receptor and ligand can occur at neutral pH, albeit to a lesser extent than at pH 5.2. This is in agreement with an investigation by Barton demonstrating that cell surface-localized TLR9 can be activated by endogenous DNA-IgG immune complexes at neutral pH (Barton 2006).

### 3.1.4 Natural ODNs Exert a Sequence-Independent Cooperative Effect on Ligand Binding

Although complexes between plasmid DNA and the TLR9(LBD) are easily resolved by AEMSA, TLR9(LBD)-ODN complexes cannot be resolved. This is a consequence of both the small size and limited negative charge of the single-stranded ODN. It was hypothesized that the binding of ODNs by TLR9 could instead be investigated based on the influence of ODNs on the interaction between plasmid and TLR9(LBD). It was anticipated that ODNs and plasmids would compete for the same

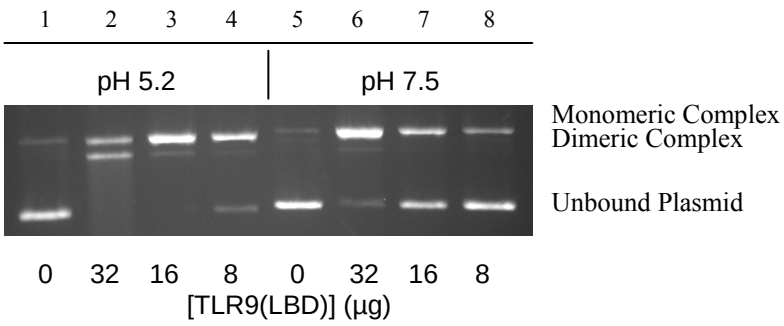



**Figure 3.3: pH Dependence of TLR9(LBD) Ligand Binding.** TLR9(LBD) is better able to bind plasmid at acidic pHs. Lane 1: CMV (1.4 μg); Lanes 2-4: CMV (1.4 μg) with 8, 16 and 32 μg of TLR9(LBD) respectively. Reactions 1-4 were buffered at pH 5.2; Lanes 5-7: plasmid CMV (1.4 μg) with 8, 16 and 32 μg of TLR9(LBD) respectively. Reactions 5-7 were buffered at pH 7.5.

binding site on TLR9. Thus, the competitive inhibition exerted by either nucleic acid molecule would indicate preferential binding by the receptor. Rather than competing with plasmid for TLR9 binding, ODNs promoted the binding of plasmid by the receptor. Nucleoprotein complex formation occurred at protein concentrations lower than those required for formation of TLR9(LBD)-plasmid complexes (**Figure 3.4a**). The cooperative promotion of nucleic acid binding was exerted in a sequence-independent fashion as activating and non-activating PD-ODNs are equally efficient in promoting this cooperative effect. This hypothesis is supported by an investigation by Latz, which demonstrated that CpG- and GpC-ODNs immunoprecipitate TLR9 with equivalent efficiency, and Roberts, who demonstrated by SPR that although TLR9 demonstrates greater affinity for CpG-ODNs, GpC-ODNs can bind to TLR9 at high concentrations (Latz 2004, Roberts 2005). The cooperative effect of ODNs on TLR9 ligand binding does not require the incorporation of the ODN into the nucleoprotein complex as $^{32}$P-labeled PD-ODNs do not co-migrate with the distinctly migrating species (data not shown).

### 3.1.5 Phosphothioate ODNs Exert Less of a Cooperative Effect on Ligand Binding than Phosphodiester ODNs

As phosphodiester ODNs are susceptible to endogenous nuclease enzymes, they are poorly suited for *in vivo* investigations of TLR9. Thus, many investigations of TLR9 utilize phosphothioate-modified ODNs (PTO-ODN). The PTO-ODN modification employs the replacement of one of the non-bridging backbone oxygen atoms with sulfur

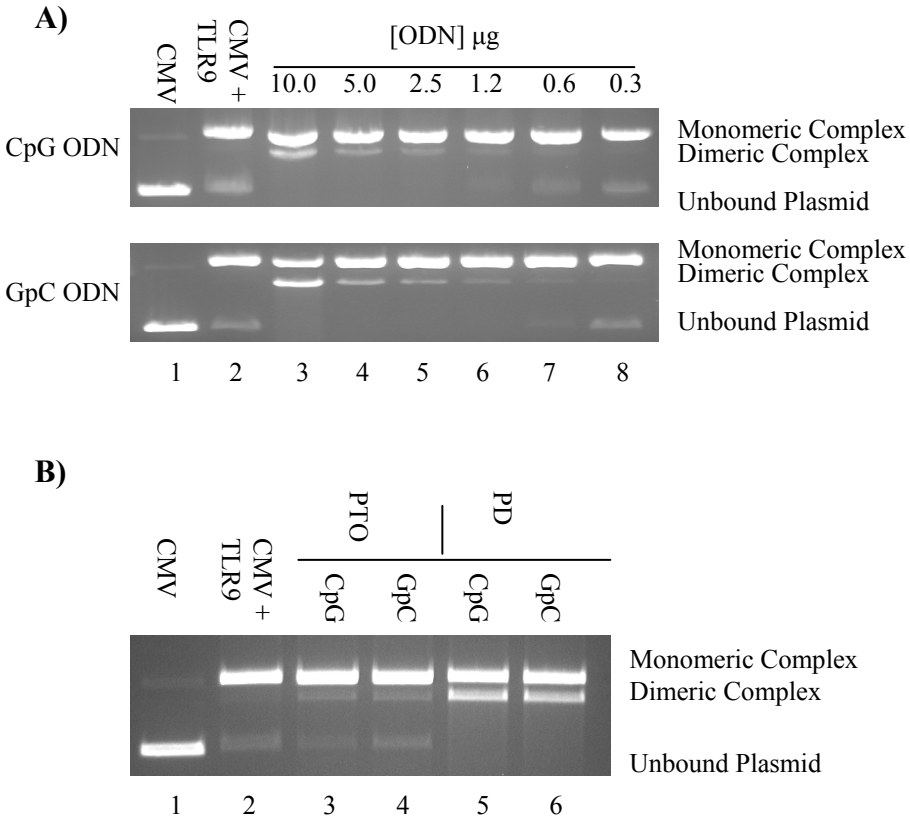



**Figure 3.4: Cooperative Influence of ODNs on Plasmid Binding. A) ODNs Exert a Sequence-Independent Cooperative Effect on Plasmid Binding.** CMV and TLR9(LBD) are used in constant quantities of 1.4 µg and 8 µg respectively. Lane 1: CMV; Lane 2: CMV and TLR9(LBD), Lanes 3-8: identical to lane 2 with the addition of serial 2-fold dilutions of either CpG of GpC PD ODN 2007 starting at 10 µg. **B) Phosphodiester ODNs Exert a More Potent Cooperative Effect than Natural ODNs** Lane 1: CMV; Lane 2: CMV and TLR9(LBD), Lane 3: CMV, TLR9(LBD) and ODN PTO-CpG 2007, Lane 4: CMV, TLR9(LBD) and ODN PTO-GpC 2007, Lane 5: CMV, TLR9(LBD) and ODN PTO-CpG 2007, Lane 6: CMV, TLR9(LBD) and ODN PTO-GpC 2007. Each ODN was present at 10 µg.

thus bestowing greater stability due to nuclease resistance. As this investigation has demonstrated that ODNs promote plasmid binding, two pairs of ODNs of identical sequence, differing in the presence of either a natural phosphodiester or a modified phosphothioate backbone, were examined for their influence on the interaction between TLR9 and plasmid DNA. In the presence of either activating or non-activating PD-ODNs, binding of plasmid DNA by TLR9 is promoted whereas neither sequence, in the context of PTO-ODNs, was able to exert this cooperative effect (**Figure 3.4b**).

The lack of cooperative influence on plasmid binding by PTO-ODNs in comparison to the PD-ODNs contrasts with the ability of PTO-ODNs to mimic bacterial DNA-induced innate immune response activation *in vivo*. That PTO- and PD-ODNs exert different influences on the binding of nucleic acids by TLR9 suggests that they are not direct functional analogs. Thus, these results emphasize the structural and functional distinction due to ODN backbone modification. These results are in agreement with reports challenging the neutrality of the PTO modification. It has been speculated that an altered specificity of interaction exists between PTO-ODNs and TLR9 and that PTO-ODNs can bind other TLR family members (Krieg 2002, Hartmann 2000, Roberts 2005).

### 3.1.6 Plasmid DNA Exerts a Cooperative Effect on ODN Binding

As phosphodiester ODNs mediates a cooperative effect on plasmid binding by TLR9, it was hypothesized that plasmid DNA would also exert the same positive effect on the binding of ODNs. AEMSA analysis relies on large, highly negatively charged complexes for efficient resolution and is therefore of limited use in visualizing ODN-

TLR9(LBD) complexes. As a consequence of these limitations, a secondary analysis was employed that would allow for resolution of TLR9-ODN complexes. Acrylamide EMSA analysis resolves nucleoprotein complexes based primarily on protein characteristics with only minor influence from the nucleic acid. Previous characterizations of the binding of transcription factors to promoter elements have confirmed the validity of this technique for visualizing DNA-protein complexes (Frontini 2001).

Stable $^{32}$P-ODN-TLR9(LBD) complexes can be resolved and visualized through acrylamide gel shift assays. Based on these assays it is apparent that TLR9(LBD) binds ODNs more efficiently in the presence of plasmids (**lane 6, Figure 3.5**). TLR9-ODN complex formation is negligible in the absence of plasmid (**Figure 3.5**).

### 3.1.7 TLR Recognizes Higher Order CpG Motifs

That CpG-, rather than GpC-, ODNs specifically activate innate immune responses in a TLR9-dependent manner has been well documented (Krieg 1999, Bauer 1999). The cell stimulation responses from these investigations suggest a sequence-dependent interaction between the receptor and the nucleic acid ligand. However, the specificity of this interaction has been called into question. Notably, investigations by Latz and Roberts demonstrate a lack of sequence specificity in nucleic acid binding by the receptor (Latz 2004, Roberts 2005).

Previous investigations have demonstrated that TLR9 is activated by higher order CpG-containing sequences in a species-specific fashion. This higher order

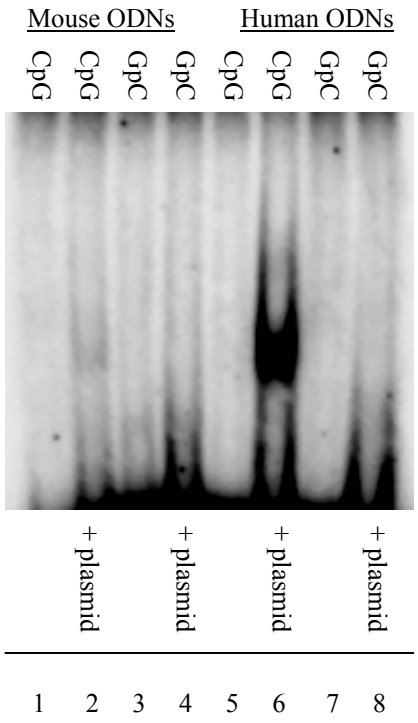



**Figure 3.5: TLR9(LBD) is Highly Sequence-Specific.** Constant quantities of CMV (1.4 μg), TLR9(LBD) (8 μg) and ODN (0.5 μg) were used in each assay and added simultaneously. Lane 1: TLR9(LBD) and mouse CpG ODN, Lane 2: TLR9(LBD), CMV and mouse CpG ODN, Lane 3: TLR9(LBD) and mouse control ODN, Lane 4: TLR9(LBD), CMV and mouse control ODN, Lane 5: TLR9(LBD) and human CpG ODN, Lane 6: TLR9(LBD), CMV and human CpG ODN, Lane 7: TLR9(LBD) and human control ODN, Lane 8: TLR9(LBD), CMV and human control ODN.

sequence, termed the CpG motif, is comprised of a hexanucleotide sequence surrounding the central CpG dinucleotide. It has been demonstrated that murine TLR9 is preferentially activated with a GACGTT sequence whereas human and bovine TLR9 respond greatest to (G/T)TCGTT. It is likely that the single base change within the human/bovine and murine CpG motifs influences TLR9 activation as a result of modification to the intimacy of the receptor-ODN interaction, although it has been speculated that human TLR9 may have less defined sequence specificity (Dalpke 2004).

This investigation demonstrates that TLR9(LBD) is able to discriminate not only CpG- and GpC-ODNs with a high-degree of efficiency, but also the higher order species-specific CpG motif. Stable nucleoprotein complex formation is greatest with ODNs containing the human CpG motif **(lane 6, Figure 3.5)**. A small amount of complex formation is also evident with murine CpG motif ODN, although this is approximately 10-fold less than that with the human CpG motif (**lane 2, Figure 3.5**). This specificity corresponds to the discrimination of ODNs that are identical at 31 of 33 positions.

### 3.1.8 Positive Cooperativity of Endogenous TLR9

The mutual cooperativity exerted by nucleic acids, in the form of ODNs or plasmids, on binding of nucleic acids by TLR9 prompted the consideration of the physiological significance of this phenomenon. Previous investigations of TLR9 activation have utilized cell proliferation assays, thus providing a valuable platform to monitor the significance of this cooperative effect *in vivo* (Krieg 1999, Bauer 1999).

Through cell proliferation assays it was demonstrated that the cooperative effect of nucleic acids on TLR9 ligand-binding is preserved *in vivo*. The magnitude of cellular responses upon co-stimulation with plasmid and either CpG-, or GpC-, ODNs was higher than the sum of the individual responses to ODN or plasmid alone **(Figure 3.6)**. This data represents the average of experiments performed in triplicate for a single animal; three animals, each tested in triplicate, gave unique magnitudes of responses although the same trend in the responses is evident. As previously noted, differences in the magnitude of TLR9-mediated responses amongst animals in animal-to-animal comparisons are typical of cell-stimulation experiments (Mena 2003). Notably, the fold induction of the responses to the co-stimulation by ODN and plasmid, as compared to the sum of the individual responses to plasmid and ODN administered separately, was quite consistent across animals. For CpG-PTO 2007 ODN the magnitude of the cooperative effect was a 2.9±0.9 (avg ± 1 std dev.) fold induction while GpC-PTO 2007 ODN mediated a 1.6±0.7 fold cooperative induction. The fold inductions represent the ratio of the response to co-stimulation with both plasmid and ODN as compared to the sum of responses to plasmid and ODN alone.

These results reflect sequence-dependent activation by TLR9 as co-stimulation with GpC-ODN and plasmid resulted in an approximately 2-fold lower induction than that of their CpG counterparts. That a more potent induction of immune responses was observed with the CpG-, rather than GpC-, ODNs reflects the cooperative effect of plasmid-mediated activation by CpG-ODNs. It is speculated that during the cell proliferation assays TLR9 is primed by the nucleic acids, leading to the formation of a sensitized form of the receptor. This higher affinity form of the receptor is sensitized for

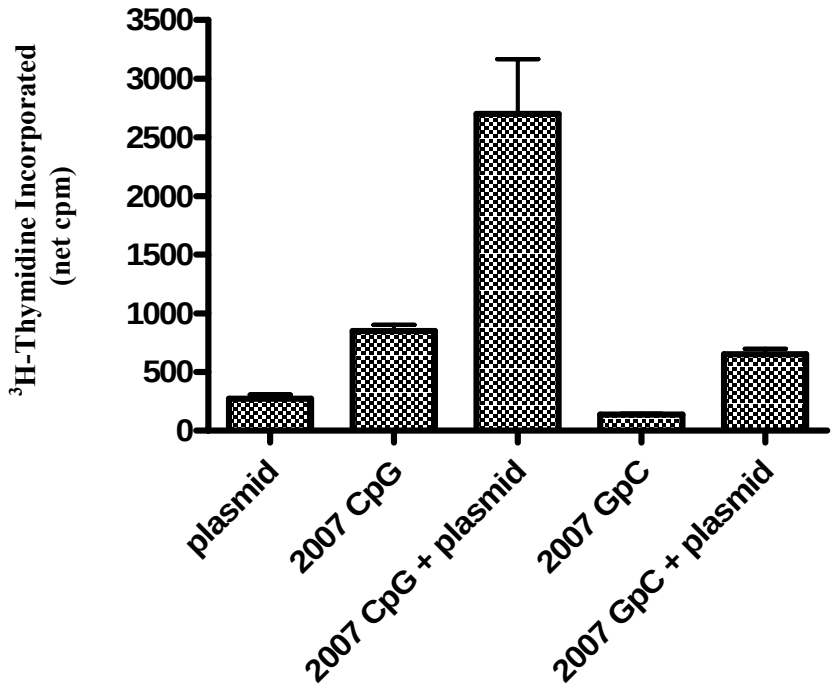



**Figure 3.6: Endogenous TLR9 Undergoes Cooperative Activation.** Bovine PBMCs (2 X $10^5$ cells/well) stimulated with either CpG or GpC PTO-ODN 2007 at 5 μg/ml, plasmid at 1.0 μg/ml or a co-stimulated with both plasmid and ODN. Cells were incubated for 72 h with $^3$H-thymidine added during the final 6 h of culture. Data presented are the mean and one standard deviation of values from triplicate cultures taken from a single representative animal. Background counts corresponding to activation by media were subtracted from all data.

binding activating (CpG) or non-activating (GpC) sequences, culminating in receptor activation or inhibition. It is also demonstrated that GpC-ODNs are able to activate TLR9, albeit to a far lesser extent than CpG-ODNs. This lends support to accumulating reports of sequence-independent binding and activation of TLR9 *in vivo*.

A point of discrepancy exists between the *in vitro* and *in vivo* assays in the context of the PTO-ODNs. In contrast to the ability of PTO-ODNs to elicit strong cooperative effects on the induction of immune responses, these modified ODNs were less efficient than PD-ODNs in mediating the cooperative effect on TLR9 *in vitro*. This discrepancy likely reflects the absolute concentration of either ODN presented to TLR9 under physiological conditions. As the cellular uptake of PTO-ODNs is approximately one order of magnitude greater than that of their phosphodiester counterparts (Sester 2000), in conjunction with the degradation of PD-ODNs by endogenous nucleases (Zhao 1993), the total concentration of ODN presented to endogenous TLR9 would be much higher when PTO-ODNs are employed in cell stimulation experiments.

### 3.1.9 Kinetics of TLR9 Activation Support a Cooperative Model

Investigations of Toll, the Drosophila homologue of the Toll-like receptors, have suggested that receptor activation is functionally influenced by both receptor-ligand and receptor-receptor interactions. Recently, Weber has demonstrated that Toll activation by Spätzle, the endogenous Toll ligand, is comprised of two non-equivalent binding events culminating in the formation of an active dimeric Toll complex (Weber 2005).

As full-length Toll ectodomains are able to undergo weak dimerization events in the absence of ligand the resting state of Toll is hypothesized to be comprised of both

ligand-free monomers and dimers (Gay 2006). In the presence of Spätzle the inactive dimers undergo interactions with the ligand and the dimer is split into ligand-bound and ligand-free monomers. Following the first binding event, Toll dimers are reformed between ligand-bound and ligand-free monomers culminating in the formation of the active Toll- Spätzle complex. Weber has demonstrated that this second binding step proceeds with an approximately three-fold lower affinity than the first binding event, suggestive of negative cooperativity (Koshland 1996).

Based on Michaelis-Menten kinetics, in the absence any of cooperative effect, an approximately 80-fold increase in substrate concentration is required to increase receptor signaling from 10% to 90% (Koshland 1996). In the event of positive cooperativity, where the subsequent binding events are favored over the first and n, the hill coefficient, is >1, less than 5-fold increases in substrate concentration are required to reach the same magnitude of activation. With negative cooperativity (n<1), where the second binding event is less favored than the first, up to 400-fold increases in substrate concentrations are required for the same increase in receptor activation. Cell stimulation experiments of Toll lend support to the proposed model of negative cooperativity by Weber as 300-500 fold increases in Spätzle concentration are required to increase signaling activity from 10% to 90% (Gay 2006) (**Figure 3.7**).

The binding of ligand by Toll through a negative cooperative mechanism has physiological implications for Drosophila. This mechanism of ligand binding results in an increased range of substrate concentrations to which Toll is responsive. A similar mechanism of activation has been reported for the follicle stimulating hormone (FSH) receptor (Urizar 2005) and the binding of nerve growth factor by p75 (He 2004).

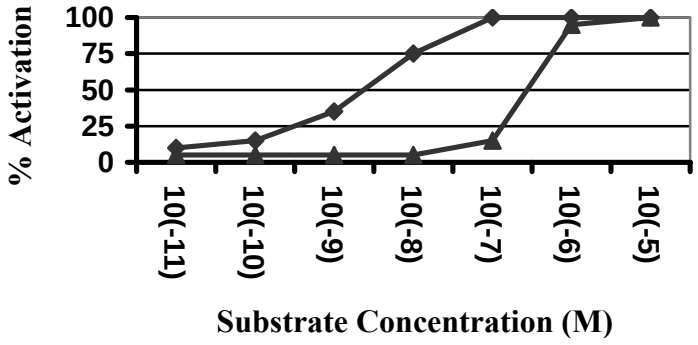



**Figure 3.7: Kinetics of Activation of Toll and Toll-like Receptor 9.** Representative data of Toll activation by Spatzle is adapted from [30]. For determination TLR9 activation, bovine PBMCs (2 X $10^5$ cells/well) were stimulated with CpG PTO-ODN 2007 at a series of concentrations. Cells were incubated for 72 h with $^3$H-thymidine added during the final 6 h of culture. Percentage activation for Toll (◆) or TLR9 (▲) are determined as a percentage of the maximal observed activation.

## 3.2 Antimicrobial Activities and Virulence Activation Potentials of Host Defense Peptides

### 3.2.1 PhoP$^-$ and PhoQ$^-$ Mutants Have Increased Susceptibility to HDPs

Traditionally, micromolar concentrations of $Mg^{2+}$ have been regarded as the activator of *Salmonella* PhoPQ. The physiological relevance of this interaction has recently been met with criticism as phagosomal $Mg^{2+}$ concentrations have been reported to be in the millimolar range and therefore PhoPQ repressive (Groisman 2006). An investigation by Bader recently demonstrated that host defense peptides can activate PhoQ and thus act as activators of PhoPQ (Bader 2003, Bader 2005).

As PhoPQ activation results in increased *Salmonella* virulence phenotypes, and HDPs are being actively pursued as novel antimicrobial therapeutics, consideration of HDPs from the perspective of bacterial virulence activation must be addressed. To investigate PhoPQ activation potentials of HDPs, a subset of peptides was selected based on a previous investigation by Hilpert (Hilpert 2005) in which direct antimicrobial activities were established for a parent peptide, Bac2A, and a series of peptide derivatives against a broad-range of bacterial species. From this investigation, a subset of peptides was selected representing derivatives with improved (R3 and Sub3), maintained (G12) or compromised (P7 and K7) antimicrobial activity, as compared to Bac2A. Modified broth-dilution MIC assays were performed for all of peptides against wild-type *S. typhimurium* and the virulence-attenuated PhoP- and PhoQ- strains. The

| | | MIC (µg/ml) | | | |
|---|---|---|---|---|---|
| **Sequence** | **Name** | **WT** | **PhoQ-** | **PhoP-** | **PhoPQ$^{comp}$** |
| GATPEDLNOKLS-$NH_2$ | NC | >500 | >500 | >500 | >500 |
| RLARIVVIRVAR-$NH_2$ | Bac2a | 32.8±4.1 | 6.2±1.6 | 6.5±1.5 | 42.5±3.5 |
| RLRRIVVIRVAR- $NH_2$ | R3 | 11.5±3.2 | 4.2±1.3 | 3.8±0.9 | 30.0±5.0 |
| RLARIVKIRVAR- $NH_2$ | K7 | 68.8±3.7 | 13.1±2.0 | 13.3±2.1 | 69.0±1.4 |
| RLARIVPIRVAR- $NH_2$ | P7 | >500 | 46.7±4.1 | 45.4±3.2 | >500 |
| RLARIVVIRVAG- $NH_2$ | G12 | 31.4±3.5 | 8.6 ±1.3 | 10.1±1.9 | 34.0±4.2 |
| RRWRIVVIRVRR- $NH_2$ | Sub3 | 10.0±2.0 | 5.7±1.0 | 6.4±0.9 | 19.0±3.8 |

**Table 3.1: Minimum inhibitory concentrations of select Bac2A peptide derivatives.** A series of peptides were screened against wild type, $PhoQ^-$, $PhoP^-$ strains of *Salmonella typhimurium* as well as the functionally reconstituted $PhoQ^-$ strain. The MIC values represent the averages of triplicate experiments.

resultant MIC values for all peptides are in close agreement with those reported previously (**Table 3.1**) (Hilpert 2005).

As anticipated, resistance to antimicrobial peptides is severely compromised in the PhoP- and PhoQ- strains. The MIC values of all peptides were significantly lower in the mutant strains than those against the wild-type strain. This is in agreement with previous investigations in which the loss of either PhoP or PhoQ resulted in ablation of bacterial virulence responses (Fields 1989). The MIC values for the peptide set demonstrate that the PhoP- and PhoQ- strains are functionally equivalent as loss of either component results in comparable decreases in MIC values for each peptide. As expected, the inability to either detect, or respond to, a particular stimulus results in functionally similar phenotypes.

Given that the removal of either PhoP or PhoQ abrogated bacterial defensive responses, it was hypothesized that the reconstitution of PhoPQ in the mutant strains would result in wild-type-like responses to antimicrobial peptides. Following cloning of the PhoPQ operon from the wild-type genome, PhoPQ was complimented within the PhoQ- and PhoP- mutant strains. Functional complementation of PhoPQ with a broad species-range plasmid (pBBR1MCS-4) expressing the PhoPQ operon ($PhoPQ^{comp}$) returned MIC values to levels comparable with the wild type strain (**Table 3.1**). The MICs against the $PhoPQ^{recon}$ strain were slightly higher for all peptides tested as compared to those against the wild-type bacterium. These results are indicative of a lower HDP susceptibility, or increased bacterial defensive responses. This is likely a consequence of higher levels of expression of the PhoQ and PhoP proteins from the plasmid as compared to wild-type levels from the endogenous PhoPQ operon.

### 3.2.2 Changes in Direct Antimicrobial Activity are a Minor Component of the HDP Antimicrobial Efficiency

The previous analysis established MIC values for the peptide set against wild-type, and PhoP- and PhoQ- strains. These MIC values are indicative of bacterial susceptibilities to antimicrobial peptides and the initiation of bacterial defensive responses. Information regarding the direct antimicrobial activity and the PhoQ ligand potential of these molecules can be extrapolated from MIC assay data.

The antimicrobial activity of a peptide against a bacterium compromised in defensive responses, such as those of the PhoP- or PhoQ- strains, is defined as the direct antimicrobial activity (dAMA). Thus, the dAMA value for a peptide is equivalent to the MIC value for the peptide against either the PhoQ- ($MIC_{Q-}$) or PhoP- ($MIC_{P-}$) mutants. As the PhoP- and PhoQ- mutants are functionally equivalent with respect to HDP sensitivity, either the $MIC_{P-}$ or $MIC_{Q-}$ values can be regarded as a measure of dAMA. The results presented here calculate dAMA values as the averages of the $MIC_{P-}$ and $MIC_{Q-}$ scores for each of the peptides.

For any peptide derivative (PD), the relative changes in direct antimicrobial activity can be quantified with respect to the parent peptide (PP) by:

$$\Delta dAMA = dAMA_{(PD)} - dAMA_{(PP)}$$

The difference between the dAMA values of a particular PD and the PP demonstrate the influence of peptide sequence alterations on direct antimicrobial activities (**Table 3.2**). Improvements in direct antimicrobial activity are noted by peptides with negative ΔdAMA scores.

| Name | dAMA | ΔdAMA | BDR | ΔBDR | ΔPE |
|---|---|---|---|---|---|
| Bac2a | 6.2±1.6 | - | 26.6±4.4 | - | - |
| R3 | 4.2±1.3 | -2.0±2.1 | 7.3±3.5 | -19.3±5.4 | -21.3±5.8 |
| K7 | 13.1±2.0 | 6.9±26 | 55.7±4.2 | 29.1±5.8 | 33.4±6.4 |
| P7 | 46.7±3.1 | 40.5±3.5 | - | - | - |
| G12 | 8.6±1.3 | 2.4±2.1 | 22.8±3.7 | -3.8±5.5 | -1.4±5.9 |
| Sub3 | 5.7±1.0 | -0.5±1.9 | 34.3±2.2 | -22.3±4.7 | -22.8±5.1 |

**Table 3.2: Calculation of changes on direct antimicrobial activity and activation of bacterial defensive responses.** The peptides R3 and Sub3 are improved antimicrobials as indicted by the decreased MIC against wild type Salmonella. This effect is largely mediated through the decreased tendency to activate PhoQ-dependent bacterial defensive responses, as indicated by the ΔBDR scores. All values are in units of μg/ml. dAMA refers to the direct antimicrobial activity, BDR represents the bacterial defensive response and PE is the peptide efficiency.

The changes in direct antimicrobial values were found to be moderate amongst the peptide set examined. The peptides R3 and K7 represent the boundaries of the ΔdAMA values at -2.0 and 6.9, respectively. This contrasts with the differences in the MICs of the same peptides against the wild type strain; MICs for R3 and K7 ranged from -21.3 and 36.0, respectively, with respect to Bac2A. As the ΔdAMA values for each of the peptides are similar, amino acid substitutions within the peptide derivatives do not have a significant effect on direct antimicrobial activity. That the largest change in direct antimicrobial activity (6.9 μg/ml for peptide K7) is nearly an order of magnitude smaller than the range of MICs against wild-type bacteria suggests that the primary functional difference amongst the peptides involves mechanisms specific to the PhoPQ system.

### 3.2.3 Activation of Bacterial Defensive Responses Contributes Significantly to Antimicrobial Efficiency

In addition to the direct antimicrobial activity of host defense peptide, the ability of a peptide to initiate bacterial defensive responses is also related to overall peptide efficiency. These bacterial defensive responses (BDR) are induced upon recognition of a particular peptide by the sensor kinase PhoQ.

The bacterial defensive response (BDR) is a measurement of the phenotypic changes initiated upon recognition of a host defense peptide. The phenotypic changes induced by the BDR decrease the ability of HDPs to interact with the bacterial membrane by way of modifications to the negative charge of the membrane and the

secretion of proteases. As PhoPQ is the primary system by which *Salmonella* initiates such defenses, the BDR is more specifically the PhoPQ-dependent alterations induced upon peptide recognition by PhoQ. Thus, BDR, although reflective of a bacterial behavioral response to host defenses, is also a characteristic of the peptide in question in terms of its ligand potential for PhoQ.

The magnitude of the BDR can be calculated by direct comparison of wild-type and mutant strain responses to peptide. Specifically, the magnitude of the BDR activated by a peptide is calculated as the difference in the MIC values between the wild type strain and either of the mutant strains:

$$BDR = MIC_{wt} - MIC_{Q-} \text{ or } BDR = MIC_{wt} - MIC_{P-}$$

As the MIC value of a peptide against the mutant strains was demonstrated to be equivalent to the dAMA value, calculation of BDR can be simplified by substitution of the dAMA value, such that:

$$BDR = MIC_{wt} - dAMA$$

As the BDR is the magnitude of PhoPQ-dependent resistance mechanisms in the presence of peptides, it can also be used for comparisons between peptide derivatives and the parent peptide:

$$\Delta BDR = [MIC_{wt(PD)} - dAMA_{(PD)}] - [MIC_{wt(PP)} - dAMA_{(PP)}]$$

As for ΔdAMA, a negative score indicates a more efficient peptide in terms of PhoQ evasion and is anticipated to reflect differences in the abilities of the peptides to serve as PhoQ ligands. Based on the ΔBDR scores, peptides R3 and Sub3 are more efficient than Bac2A at evading the PhoPQ system; the ΔBDR for R3 and Sub3 were -19.3 and -22.3, respectively (**Table 3.2**). In contrast, peptide K7 had an improved ability to activate PhoPQ with a ΔBDR score of 29.1. Thus, peptide K7 represents an

improved PhoQ ligand with the propensity to induce bacterial defensive responses whereas peptides R3 and Sub3 are improved in evasion of PhoQ.

### 3.2.4 Relative Contribution of Changes in Direct Antimicrobial Activity and Bacterial Defensive Responses to Antimicrobial Efficiency

The overall change in the net efficiency of a peptide derivative (ΔPE) is taken as the change in MIC against the wild type bacteria. ΔPE can be expressed as a function of the contributions of alterations to direct antimicrobial activity and as well alterations to bacterial defensive response activation:

$$\Delta PE = \Delta dAMA + \Delta BDR$$

Negative scores indicate improved peptides where the magnitude of the score reflects the degree of improvement. Notably, the ΔPE score mirrors the change in MIC between a parent peptide and its derivative against wild-type bacteria. The advantage of this system is that it allows quantification of the relative changes in each of these peptide behaviors.

Two of the peptides, R3 and Sub3, have significant reductions in their tendency to initiate PhoQ-dependent bacterial defensive responses as indicated by their ΔBDR scores of -19.3 and -22.3, respectively. The magnitude of these differences corresponds very closely with the observed difference in MIC against wild type bacteria. This suggests that the bacterial defensive response component is a principle determinant for changes to antimicrobial efficiency. Relative to the Bac2A parent peptide, the R3 peptide demonstrates improved antimicrobial activity against wild-type bacteria with a

reduction in the MIC by -21.3 μg/ml. As a large proportion of this (-19.3 μg/ml) can be accounted for by the reduced bacterial defensive response, it appears that the improved efficiency of these peptides is determined almost exclusively by the evasion of PhoQ (**Figure 3.8**, **Table 3.2**).

In contrast, the K7 peptide elicits a much stronger defensive response than the parent peptide Bac2A, as demonstrated by a ΔBDR score of 29.1. This implies that the K7 peptide is better able to function as a ligand for the PhoPQ system and, as a consequence, has reduced antimicrobial efficiency. The overall change in direct antimicrobial activity for peptide K7 (6.9 μg/ml), and the tendency of this peptide to induce bacterial resistance phenotypes, contribute to the dramatic reduction in peptide efficiency for the K7 peptide. Based on the ΔBDR and ΔdAMA scores, it appears that the tendency of K7 to initiate bacterial defensive responses is the most significant determinant in defining its efficiency (**Figure 3.8**, **Table 3.2**).

### 3.2.5 Changes in the Bacterial Defensive Response Correlate with the Ability to Activate PhoPQ

Thus far, the primary conjecture has been that bacterial defensive responses induced during host defense peptide challenge reflect the differential PhoQ-ligand potentials of the peptides. As the calculations have been based on direct comparisons between wild-type and PhoPQ deletion strains, this would seem a valid assumption. Nevertheless, the PhoPQ regulon is complex with the ability to modulate a wide variety

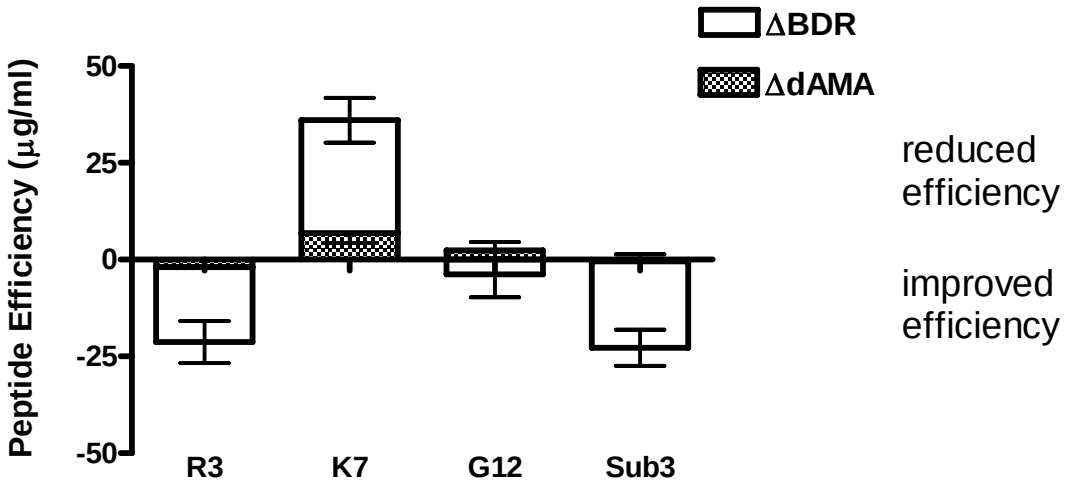



**Figure 3.8: Net Peptide Efficiencies for Bac2a peptide derivatives.** Relative contribution of changes in direct antimicrobial activity and tendencies to activate bacterial defensive responses towards the change in net peptide efficiency of a peptide derivative as compared to the parent peptide. Peptides with negative scores indicate more effective antimicrobials.

of bacterial behaviors, as well as the ability to influence the workings of other TCSs such as PmrAB, implicated in resistance to certain antimicrobial peptides in *Salmonella* and other strains (Bader 2003, McPhee 2003, Tamayo 2002). Thus, direct verification of bacterial defensive response activation through a PhoQ-HDP interaction was sought.

Previously, Miller demonstrated that activation of the PhoPQ system *in vivo* could be quantified through an *S. typhimurium* reporter strain in which expression of an acid phosphatase is regulated by PhoP activation (Miller 1989). Through this reporter system, the ability of sub-lethal concentrations of the peptides to activate PhoPQ was determined to be a unique characteristic for each peptide. Importantly, the degree of PhoPQ activation by the peptides closely correlates with the patterns of the peptide BDR scores. The peptide K7, which was the least effective antimicrobial peptide, was the most potent PhoQ ligand, initiating PhoQ activation approximately 2.3-fold higher than what is observed under repressing concentrations (5 mM) of magnesium (**Figure 3.9**). It is of note that the magnitude of activation by peptide K7 is comparable to that of activating $Mg^{2+}$ concentrations (50 μM). The activation of PhoQ by the K7 peptide was approximately 80% that induced following micromolar magnesium incubation (**Figure 3.9**). This is in accordance with levels of HDP-mediated PhoQ activation reported previously (Bader 2005). Conversely, the most effective antimicrobial peptides, R3 and Sub3, initiated the lowest levels of PhoPQ activation which is consistent with their low BDR scores (**Figure 3.9)**. The results of the alkaline phosphatase assays verify that the peptides predicted to be the best PhoQ ligands through the MIC analysis are indeed the best activators of PhoQ *in vivo*.

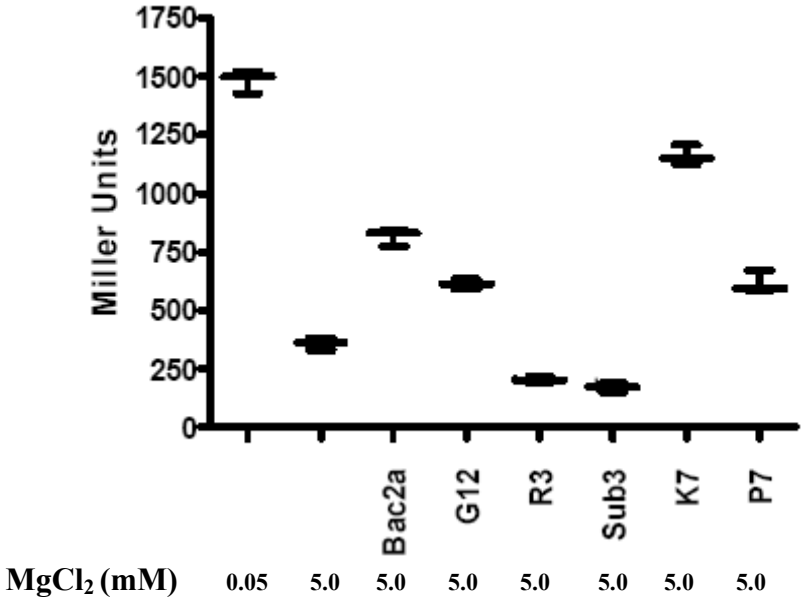



**Figure 3.9: *In vivo* Activation of PhoPQ by Low Magnesium and Host Defense Peptides.** The ability of Bac2A peptide derivatives to initiate activation of a PhoPQ-dependent reporter was examined by incubation of bacterium in the presence of sub-lethal (1 ug/mL) concentrations of indicated peptide. Values reported represent the averages of three replicates. Activation and repression of PhoPQ by low (50 μM) and high (5 mM) magnesium respectively is also reported.

### 3.2.6 Exposure to Sub-lethal Concentrations of HDPs Increases Resistance to a Broad Spectrum of Host Defense Peptides

Concerns have been recently raised that the therapeutic applications of sub-lethal concentrations of host defense peptides might result in persistent infections by induction of HDP-resistant bacterial phenotypes. These concerns prompted the examination of sub-lethal HDP concentrations as inducers of bacterial HDP resistance mechanisms by way of PhoPQ activation.

To determine if exposure to sub-lethal concentrations of HDPs initiates bacterial defensive responses, and if the magnitude of these responses correlates with the calculated BDR scores, bacteria were pre-incubated with an increasing sub-lethal concentration of either the K7 or R3 peptides over a 4-hour time period. These peptides represent the two extremes of peptide efficiencies examined in the peptide derivative subset in terms of the calculated BDR scores and thus their ability to promote (peptide K7) or avoid (peptide R3) bacterial defensive responses. Following this pre-adaptive period, bacteria were subjected to standard MIC assays. The protective effects mediated by the pre-adaptive exposure to K7 or R3 were quantified based on the MIC values. Consistent with the calculated BDRs, the K7 had the most dramatic shift in MICs scores, corresponding to up to 10-fold increases over the values observed for naïve bacteria. In contrast, pre-incubation of bacteria with peptide R3 had limited impact on HDP protection with many of the MICs comparable to those observed for naïve bacteria (**Figure 3.10**). This demonstrates that peptides registering the greatest BDR scores also mediate the greatest tendency for induction of adaptive resistance phenotypes within bacteria.

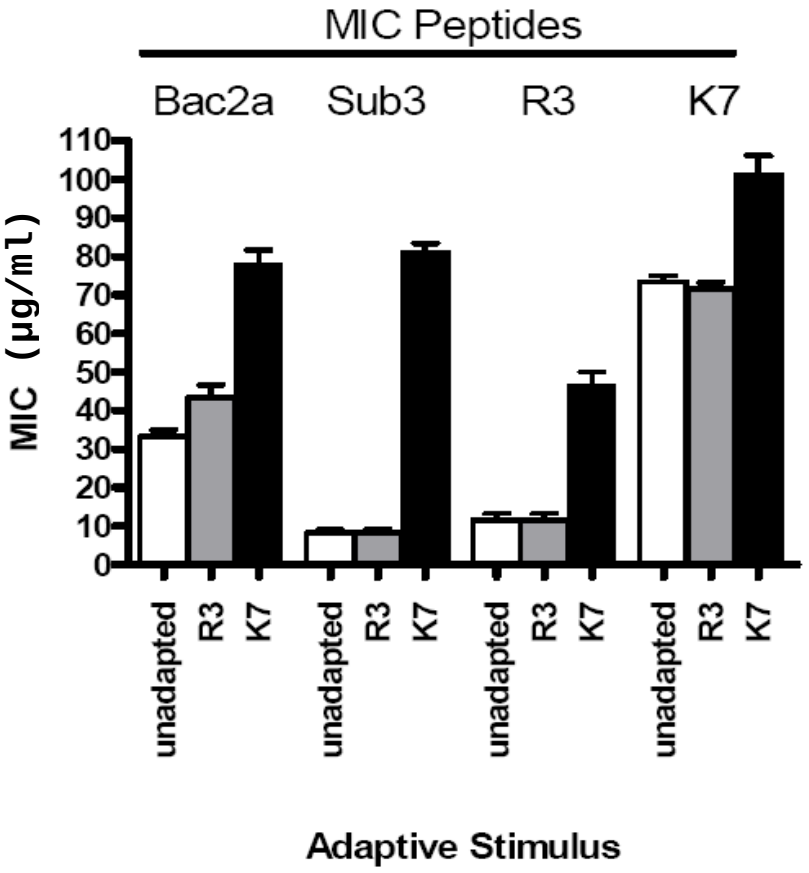



**Figure 3.10: Activation of HDP Responses Following Exposure to Sub-Lethal Concentrations of HDPs.** The ability of peptide derivatives to induce adaptive responses was determined by pre-incubation of bacteria for four hours in the presence of sub-lethal concentrations (5 ug/mL) of peptides prior to standard MIC assays. Values reported represent the averages of three repeats.

### 3.2.7 RI-BMAP28 is Resistant to Proteolytic Degradation

The therapeutic administration of host defense peptides has been limited by the high manufacturing costs of peptides and low systemic stabilities; single dosage costs of HDPs are approximately 100 times more expensive than that of conventional antibiotics (Marr 2006). Thus, an alternative strategy is presented that focuses on the increased stability of HDPs through the introduction of retro-inverso modification. This modification is reminiscent of the phosphothioate modification in PTO-ODNs.

Retro-inverso peptide modification involves the inversion of the amino acids by incorporation of the D-enantiomers and reversal of the amide bonds. Previously, increased peptide stability due to the inversion of the natural L-amino acids to the D-enantiomers has been reported (Chen 2006). As the immunomodulatory effects of host defense peptides rely on their ability to be recognized by host biomolecules, chirality of the peptide must be maintained to allow for proper recognition events. The antimicrobial activities of HDPs rely on the interaction of these molecules with the achiral bacterial membrane and therefore requires that the only the overall three-dimensional conformation of the peptide must be maintained (alpha-helix, beta-sheet, and extended).

The modification of BMAP28 by retro-inversion results in a large increase in protease resistance. As BMAP28 and RI-BMAP28 are rich in lysine and arginine residues, the serine protease trypsin was selected for analysis. Within 30 minutes of protease exposure, all of the BMAP28 peptide was cleaved at least once by trypsin, verifying that the protease lability of natural HDPs is a concern in systemic applications.

In direct contrast, the RI-peptide remained nearly completely intact throughout the 8-hour trypsin exposure (**Figure 3.11**).

### 3.2.8 BMAP28 and RI-BMAP28 Adopt Similar Conformations

The maintenance of the structural and functional characteristics of natural host defense peptides is the primary concern for the use of retro-inverso modifications in host defense peptide-based therapeutics. CD spectroscopy provides a method for determination of the elements of secondary structure within peptides and proteins and allows for the mimicking of physiological environments, such as the hydrophobic environment of the cell membrane, during analysis.

It is well documented that inverso peptides (containing all D-amino acids) are mirror images of their natural counterparts (Chen 2006). However, it cannot be assumed that retro-inverso peptides and natural peptides adhere to the same principle as identical higher order structure cannot be assumed (Fischer 2003). Retro-inverso modification alters mainchain hydrogen bonding patterns, as well as dipole moments, that are dependent upon the direction of hydrogen bonds. Thus, an alternate structure may be favoured. Previously, CD investigations have shown that natural peptides and their RI-counterparts may adopt identical higher order conformations (Merrifield 1995).

CD spectroscopy was employed to investigate the maintenance of secondary structure upon retro-inversion of BMAP28. Both the natural and RI-peptides were found to have highly disordered structures under hydrophilic conditions (results not shown). In aqueous solutions, HDPs tend to adopt random structures and form more defined tertiary structures when in contact with a hydrophobic environment (Merrifield

A)

| HDP | Sequence |
|---|---|
| BMAP-28 | GlyLeuArgSerLeuGlyArgLysIleLeuArgAlaTrpLysLysTyrGlyProIleIle ValProIleIleArgIleGly |
| RI-BMAP-28 | Gly(D)Ile(D)Arg(D)Ile(D)Ile(D)Pro(D)Val(D)Ile(D)Ile(D)Pro Gly(D)Tyr(D)Lys(D)Lys(D)Trp(D)Ala(D)Arg(D)Leu(D)Ile(D)Lys (D)ArgGly(D)Leu(D)Ser(D)Arg(D)LeuGly |

B)

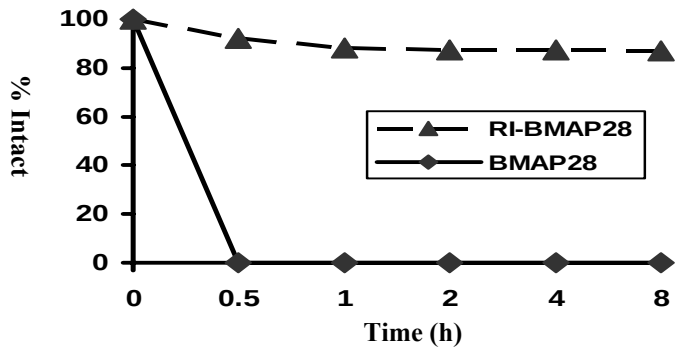


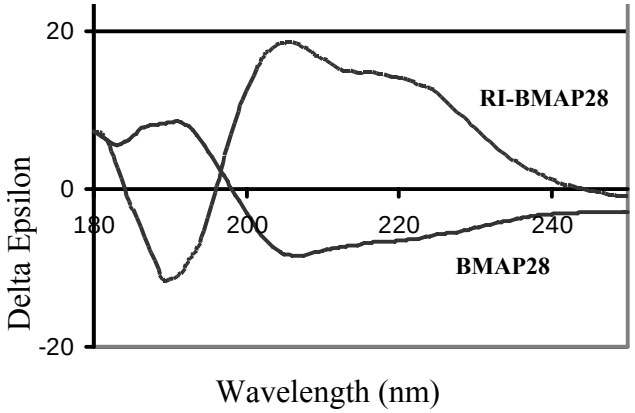

**Figure 3.11: RI-BMAP-28 is Resistant to Proteolytic Degradation.** A) Sequence of BMAP-28 and RI-BMAP-28. The RI-BMAP28 is the reversed sequence of BMAP28. B) BMAP-28 and RI-BMAP-28 (1 mg/mL) were digested with trypsin (0.1 mg/ml) in a 50 μL reaction volume (50 mM Tris pH 7.2) at 37 °C. Digestion mixtures were then separated via HPLC chromatography and the extent of peptide degradation quantified through comparison of peak areas to that of an undigested sample of the same peptide.

1995). To mimic the hydrophobic environment of the bacterial cell membrane, trifluroethanol (TFE) was incorporated with the peptide samples prior to CD spectroscopy. TFE has been used extensively in predictions of membrane-induced structural changes within host defense peptides (Lequin 2003). The CD spectra demonstrate that BMAP28 and RI-BMAP28 are relative mirror images of one another (**Figure 3.12**). Deconvolution of the CD spectra verifies that the formation of secondary structure is highly similar in both BMAP28 and RI-BMAP28 and is relatively comparable to those previously reported by others for BMAP28 (Skerlavaj 1996). Thus, the retro-inverso modificatied BMAP28 forms a highly comparable higher order structure to the natural BMAP28 counterpart.

### 3.2.9 RI-BMAP-28 has Improved Direct Antimicrobial Activity

The potential of retro-inverso peptides as novel therapeutics hinges on the maintenance of natural peptide functions following modification. Thus, the antimicrobial activity for RI-BMAP28 was compared to natural BMAP28 against a broad-range of Gram-negative bacterial species by the modified broth microdilution MIC assays. The spectrum of bacterial species used in this investigation removes biases that might be found when using smaller species samples. Across all species tested, the RI- BMAP28 exhibited a more potent antimicrobial activity than the natural peptide; MICs values for the RI-BMAP-28 were typically a half to a third of that of the natural peptide (**Table 3.3)**. As both Gram-negative and Gram-positive bacterial species secrete proteases as an endogenous defense mechanism, it is likely that the increased antimicrobial activity is reflective of higher maintained concentrations of peptide in the

A)




B)

| | BMAP-28 | RI-BMAP-28 |
|---|---|---|
| **Helical** | 13.4 | 9.9 |
| **Antiparallel** | 27.7 | 31.1 |
| **Parallel** | 10.5 | 11.1 |
| **B-Turns** | 15.8 | 14.7 |
| **Random** | 32.6 | 33.1 |

**Figure 3.12: RI-BMAP-28 Adopts Similar Secondary Structures as BMAP-28.** A) CD Spectra of BAMP28 and RI-BMAP-28. Notably, the two spectra are similar, although opposite, images. B) Percentage Composition of Different Elements of Secondary Structure.

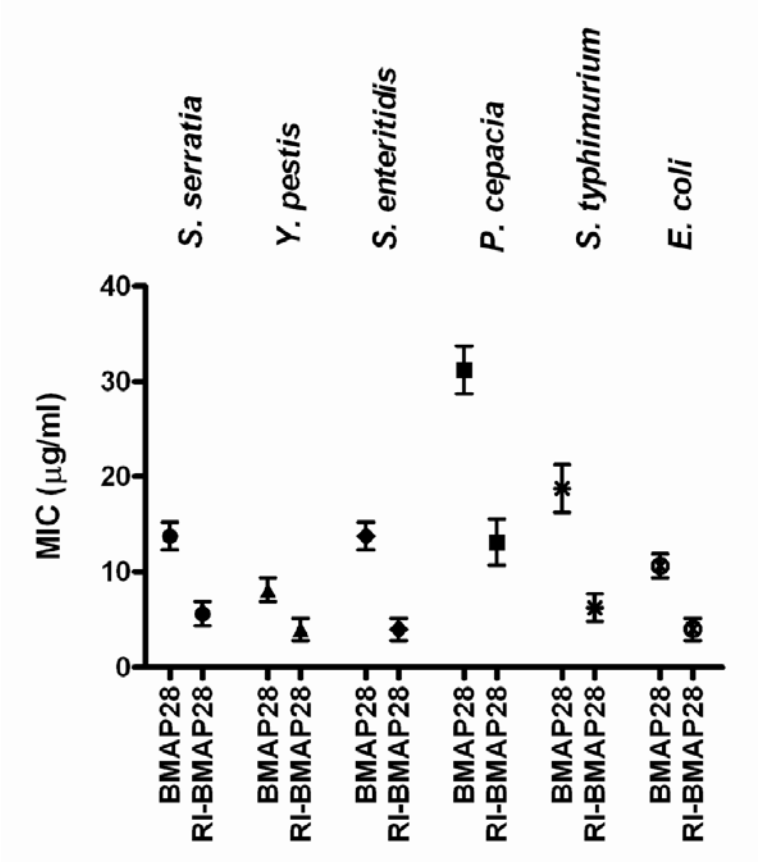



**Table 3.3: Minimum inhibitory concentrations of BMAP28 and RI-BMAP28:** The MIC values represent the averages of triplicate experiments and are in μg/ml concentrations.

MIC assay using the RI-variants. While others have reported maintained or improved direct antimicrobial activity of host defense peptides as a consequence of either retro- or retro-inverso modifications, the effects were often specific to the particular strain of bacteria (Merrifield 1995). This investigation demonstrated no strain specificity to the improved activity of RI-MAP-28 with any of the examined bacteria and is consistent with the maintained sequence and overall structure of the RI-BMAP28.

### 3.2.10 Retro-inversion of BMAP28 Reduces Cytotoxic Activity

A requirement for the therapeutic application of host defense peptides is low host cell toxicity within the range of concentrations required for therapeutic effects. Although eukaryotic membranes represent inferior targets for HDPs due to a lack of anionic lipids, absence of strong membrane potential gradient, and presence of cholesterol, it has been demonstrated that some HDPs do possess hemolytic activity (Hancock 1998, Peschel 2006, Yeaman 2003). To be considered for therapeutic administration, RI-modified peptides must not demonstrate significant hemolytic activity.

To determine the cytotoxic effects of BMAP-28 and RI-BMAP-28 the peptides were incubated overnight at a range of concentrations with bovine peripheral blood mononuclear cells. Consistent with previous investigations, BMAP-28 exerts cytotoxic effects at concentrations as low 50 μg/mL and resulted in nearly complete lysis of host cells by 250 μg/mL (**Figure 3.13**). In contrast RI-BMAP28 does not induce lysis of host cells at concentrations as high as 250 μg/mL. Thus, the RI-modification does not

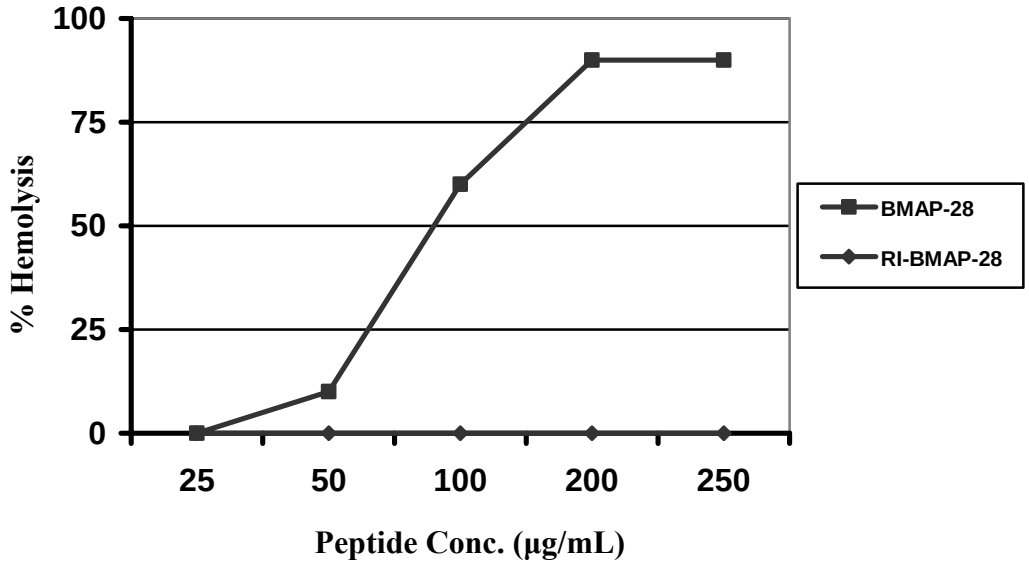



**Figure 3.13: Cytotoxicity of BMAP-28 and RI-BMAP-28.** Hemolysis was performed by incubating a 1% (v/v) suspension of bovine erythrocytes in phosphate buffered saline (pH 7.4) with a series of peptide concentrations for 12 hours at 37 C. Samples were then centrifuged and the supernatant absorbance read at 415 nm. Total hemolysis was obtained by resuspending in cells in water rather than PBS. Results are the average from four independent experiments.

conserve the hemolytic activity of BMAP28 and lends support for the use of this modification in therapeutic HDP applications. The precise mechanism by which BMAP28 mediates host cell lysis, and by which it is ablated in RI-BMAP28, has not been identified but is being investigated.

### 3.2.11 RI-BMAP-28 is Equally Effective as BMAP-28 in Suppressing LPS-Induced TNF-α Release

Bacterial infections have associated medical risks that not only include the presence of a pathogenic organism within the host but also the potential for self-induced pathology. The excessive inflammatory response induced during such a bacterial infection can result in significant host tissue damage and sepsis. To confirm that the immunomodulatory activity of BMAP28 was conserved in the RI-derivative, the ability of the two isomers to influence secretion of the proinflammatory cytokine TNF-α following LPS stimulation was examined. The secretion of TNF-α as a biomarker of inflammation responses is well established.

Previous studies have suggested that the antiendotoxic activity of cationic peptides results in part from LPS chelation as this interaction is proposed to block the interaction of LPS with serum LBP (Bowdish 2005a). However complexes between HDPs and LPS tend to be of relatively modest affinities when compared to strength of interaction between LBP and LPS (Finlay 2004). Furthermore, evidence has been presented that the suppression of LPS-induced, pro-inflammatory responses by endogenous cathelicidin LL-37 involves a variety of mechanisms independent of direct

binding to LPS (Bowdish 2005b, Murakami 2002). That LL-37 and BMAP-27 retain the majority of their anti-endotoxin activity when added 30 minutes after LPS stimulation, even after removal of LPS from the extracellular media, indicates these peptides at least partially suppress LPS-induced, proinflammatory responses by mechanism other than direct binding to LPS.

ELISA assays confirm that the ability to suppress LPS-induced TNF-α secretion is conserved in both BMAP-28 and RI-BAMP28 (**Figure 3.14**). Importantly, the peptide concentration required to mediate this effect is consistent with that required to influence patterns of gene expression as demonstrated in ongoing analysis within the Napper lab and is supportive of the biological significance of this observation (personal communication).

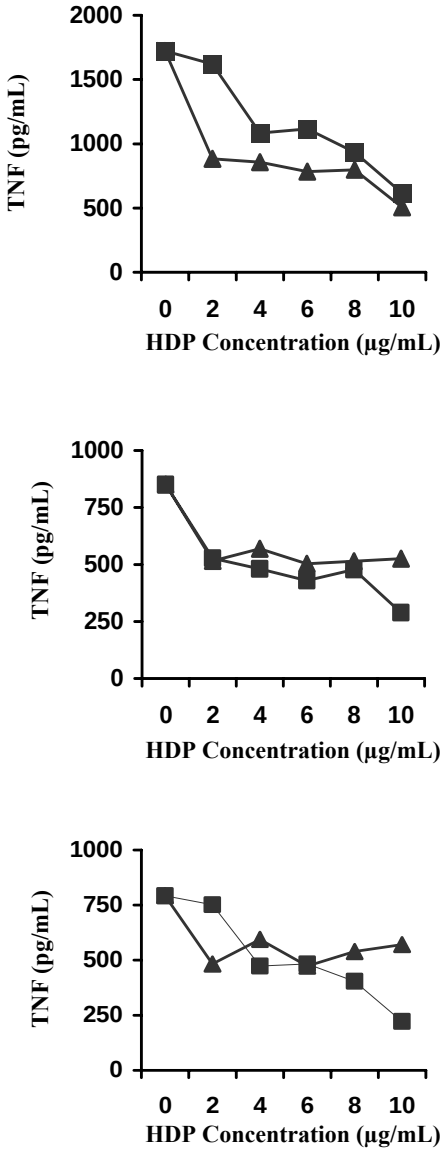



**Figure 3.14: HDP Inhibition of LPS-Induced TNF-α Secretion.** Bovine PBMCs (5 X $10^5$ cells/well) from three donors were stimulated with 100 ng/ml of LPS (Sigma) for 48 h. The secretion of TNF in culture supernatants was quantified with a capture ELISA. Inhibition of LPS-induced TNF secretion was evaluated by adding various concentrations of BMAP-28 (▲) or retro-inverso RI-BMAP-28 (■) to duplicate PBMCs cultures.

# 4.0 Discussion

## 4.1 TLR9 Interacts With Nucleic Acid Ligands in a Sequence-Independent Fashion but Activation is Sequence-Dependent

Throughout millions of years of co-evolution, the interactions between hosts and pathogens have resulted from mutual offensive and defensive strategies. From the perspective of the pathogen, these strategies have involved mechanisms for increasing the colonization of host tissues and increased evasiveness of the host innate immune arsenal. To this end, microbes rely on two-component sensory systems, such as PhoPQ, for increasing virulence characteristics within specific physiological niches. On the other hand, the host has evolved strategies that prevent such colonization through defensive early warning recognition systems and offensive antimicrobial molecules. These defensive pathogen recognition receptors are responsible for the differentiation of self from non-self. Consequently, the PRRs are responsible for the induction of an appropriate response following pathogen encounter and, ultimately, the resolution of infection.

Recently, considerable pharmaceutical interest in the innate immune system has been generated due to the potential for therapeutic enhancement and augmentation of innate immune responses. In particular, therapeutic developments of novel therapeutics based on innate immune system components have focused on TLR9 agonists and

antagonists and the cationic host defense peptides. Novel TLR9 agonists and antagonist are being investigated as therapeutics for a broad-range of malignancies. These synthetic nucleic acid TLR9 ligands are particularly attractive due to low associated costs of production, manufacturing simplicity, and low systemic toxicites (Klinman 2004). Unfortunately, there is limited structural information regarding the direct interaction between TLR9 and ligands. Thus, the development of synthetic TLR9 ligands must instead be based on the ability of these ligands to activate or repress innate immune responses in cell proliferation assays. Although cell stimulation experiments provide valuable information regarding the *in vivo* interactions between biomolecules based on global cellular responses, a failure of this approach is in the reduction of complex physiological responses to a single output. This reductionist approach cannot provide information on the discrete events such the direct interactions between a receptor and ligand. On the other hand, investigations of the direct interaction between TLR9 and DNA have also met limitations. Biochemical characterizations of this event have been limited due to difficulties associated with purification of sufficient quantities of the receptor for analyses and as well the lack of a convenient screening assay. A high-throughput screening assay that could monitor and characterize the binding interaction between TLR9 and DNA would be highly advantageous.

Several key issues surrounding the interaction between TLR9 and DNA have remained unanswered. Thus, the cornerstone of this investigation has been the following: 1) the efficiency of the receptor in binding double-stranded DNA, 2) the absolute requirement for CpG motifs within nucleic acids to mediate ligand binding and cellular activation, 3) the ability of TLR9 to recognize higher order motifs in a species-specific fashion, 4) the influence of the commonly utilized phosphothioate modification

on receptor binding, and 5) the influence of higher order allosteric mechanisms, such as those recently been reported for Toll on ligand binding . In this investigation, a novel high-throughput assay system has been proposed for the characterization of the ligand binding event - the agarose electrophoretic mobility shift assay.

Recent investigations of the TLR9 ligand binding event have utilized Surface Plasmon Resonance technology to address TLR9 ligand-binding proclivities. The investigations by Rutz and Cornelie have demonstrated a preference for CpG-, rather than GpC-, ODNs, but lacked consensus on the ability of the receptor to associate with double-stranded ligands (Rutz 2004, Cornelie 2004). Limitations in both the experimental methodology and the mode of analysis may have contributed to these discrepencies and support the methodology of this investigation.

The Cornelie and Rutz investigations employed insect cell expression systems for the expression of recombinant TLR9 protein. Although the use of insect cells allows for post-translational modifications similar to those of the endogenous receptor, the glycosylation patterns of the recombinant receptor deviate from those found under normal conditions. In particular, insect cell expression results in glycans lacking the high mannonse content normally found in mammalian N-linked carbohydrate (Tomiya 2004). Investigations of TLR3, another endosomal TLR involved in recognition of microbial nucleic acids, have noted such inconsistencies (Bell 2005). Indeed, TLR3 glycosylations have been reported to be a primary contributing factor to the binding of double-stranded RNA (Sun 2006). As structural features are well conserved amongst the subcellular TLRs, it would be hypothesized that the glycosylations found on TLR9 would also influence ligand binding. This investigation has circumvented the problems associated with previous TLR investigations through the use of a bovine expression

system. Although differences in glycosylations patterns have been noted amongst mammals, it is expected that species phylogenetically close to humans will have common elements of the glycosylations machinery (Jenkins 1996). As bovine cells were employed for expression of recombinant TLR9, the glycosylation pattern of the receptor should closely match those of the endogenous receptor therefore conserving the influence of these PTMs on TLR9 ligand binding to a high degree. Another advantage of this expression system is that the recombinant protein is secreted into the extracellular media following expression. Thus, cell lysis steps are not required with this protocol. The removal of cell lysis decreases the potential for copurification of contaminating proteins that may influence receptor characterization. In contrast, the investigations by Cornelie and Rutz utilized expression systems requiring cell lysis steps. Removal of potential contamininating proteins with wash steps were however were not reported in either protocol prior to characterization (Rutz 2004, Cornelie 2004). This is significant as cellular proteins possessing DNA-binding properties could result in false positives during the binding analysis. This may reflect the lack of consensus between the two investigations regarding the binding of single- and double-stranded DNA ligands. Additionally, SPR analyses require the immobilization of either the receptor or ligand. As immobilization limits the sampling of multiple conformations by the bound molecule it may also influence the interaction between a receptor and ligand. This limitation is removed with AEMSA and the natural receptor-ligand interaction dynamics are maintained.

That plasmid DNA has been demonstrated to activate innate immune responses (Krieg 1995), and time-course experiments have verified that the endosomal-stability of plasmids would allow for an interaction with TLR9 (Bennett 1985), suggest that it may

act as a TLR9 ligand. Investigations of the binding of plasmids by TLR9 have reached opposing conclusions regarding this event. An investigation by Cornelie demonstrated efficient binding of plasmid DNA whereas Rutz noted only low-affinity binding to double-stranded ODNs (Cornelie 2004, Rutz 2004). The results presented here validate the observations by Cornelie as the ligand-binding domain of TLR9 bound plasmid DNA efficiently. It is hypothesized that the nucleoprotein complexes formed during AEMSA correspond to monomeric and dimeric forms of TLR9 (**Figure 3.2**). Dimerization events are a recurring theme amongst the TLR family and Toll (Gangloff 2004, Weber 2005). It is also noted that the recombinant receptor demonstrates a preference for plasmids in a supercoiled conformation. This accounts for the discrepancy between the Rutz and Cornelie investigations. The linear double-stranded ODNs utilized by Rutz would be devoid of supercoiling and based on the AEMSA analysis would represent low affinity TLR9 ligands. That TLR9 can undergo interactions with both single-stranded DNA molecules and plasmids would be anticipated. Bacterial lysis within the phagosome would result in significantly high local concentrations of both molecules in proximity to TLR9.

A recurrent theme amongst the intracellular-localized Toll-like receptor family is a pH-dependency on ligand binding. Investigations of TLR3 and TLR8, have demonstrated that ligand binding and receptor dimerization are dependent on the acidic pH of the endosome (de Bouteiller 2005, Gibbard 2006). That inhibitors of endosomal acidification ablate immune responses to CpG-ODNs suggest a similar pH dependency for TLR9 (Hacker 1998, Yi 1998, Yoshimori 1991, Macfarlane 1998). The SPR analysis by Rutz is also supportive of this (Rutz 2004). AEMSA analysis confirms that the recombinant receptor formed and maintained specific nucleoprotein complexes most

efficiently at a pH comparable to that of late endosomes (**Figure 3.3**). It is also of note that the receptor can form nucleoprotein complexes at neutral pH, although at much lower efficiency. Although the stability of nucleoprotein complex formation is limited at neutral pH it may result in TLR9 activation and the initiation of innate immune responses. This is supported by a growing number of reports detailing the ability of TLR9 to be activated by host DNA (Leadbetter 2002, Boule 2004, Means 2005, Barton 2006).

The generally accepted paradigm of TLR9 activation postulates that unmethylated CpG motifs are the specific ligands for the receptor. The vast majority of supportive evidence for this model has been inferred from cell stimulation experiments. Although the sequence-dependent activation of TLR9 has been assumed to represent sequence-specific binding by the receptor, recent *in vitro* investigations have lacked consensus with regards to the sequence-specific binding of nucleic acids by TLR9 (Cornelie 2004, Rutz 2004 , Yasuda 2006). The demonstration that the sera of SLE patients can activate TLR9 also questions the paradigm of sequence-dependent receptor activation as CpG dinucleotides are both repressed in frequency and highly modified within the vertebrate genome (Leadbetter 2002, Boule 2004, Means 2005).

Although the direct interaction between TLR9 and ODNs cannot be investigated by AEMSA, the influence of these molecules on the binding of plasmid DNA by the receptor can. It is evident that both CpG- and GpC-ODNs increase the propensity of TLR9-plasmid complex formation (**Figure 3.4**). This is likely due to promotion of TLR9 to a sensitized, higher affinity form by the ODNs, resulting in an increased affinity of the receptor for binding nucleic acids of activating or non-activating

sequences. The cooperative effect of ODNs on plasmid binding was also confirmed to be independent of their incorporation into the TLR9-plasmid complexes.

Surprisingly, this cooperative influence was largely ablated upon phosphothioate modification of the ODN backbone. As PTO-ODNs are nuclease-resistant analogs of their phosphodiester counterparts, active pursuit of novel TLR9 therapeutics have focused on these modified ligands. That the PTO modification is demonstrated to functionally influence the interaction of ODNs with TLR9 argues for more thorough investigations of this influence. Thus, the development of novel TLR9 therapeutics must consider the effects of this stabilizing modification on TLR9 interactions at the level of ligand binding. Supporting this, Roberts has demonstrated that PTO- and PD-ODNs display non-equivalent binding affinities for TLR9 *in vitro* (Roberts 2005). PTO-modified ODNs have also been observed to undergo non-specific interactions with other TLR family members (Rutz 2004). This is particularly concerning as the over-stimulation of the innate immune system through the activation of multiple TLRs could result in tissue damage or sepsis (Bishop 2006).

A discrepancy exists between the influence of PTO- and PD-ODNs on plasmid binding by TLR9 *in vitro* and the activation of innate immune responses by both types of ODNs *in vivo.* Although *in vitro* PD-ODNs exert a far greater cooperative effect on TLR9 binding than their PTO counterparts, the endosomal concentration of PD-ODNs required for this cooperative influence would be exceedingly difficult to attain *in vivo* via direct administration. Indeed, analyses of TLR9 activation by PD-ODNs have utilized repeated administrations of the ligand to the growth media in an attempt to counterbalance losses due to nuclease degradation (Elias 2003, Zelenay 2003). In contrast, analyses utilizing PTO-ODNs require only single administrations. These

discrepancies must however be tempered with an appreciation for the differences between cell stimulation experiments and the normal course of bacterial invasion. Microbial DNA, although susceptible to degradation by nuclease enzymes, are encapsulated within the microorganism therefore protecting these TLR9 ligands from degradation by nucleases in the extracellular matrix. Following phagocytosis of a bacterium, high concentrations of the CpG-rich microbial DNA would be liberated within the phagosome and in proximity to TLR9.

In contrast to the sequence-independent functional influence of PD-ODNs on plasmid binding by TLR9, the formation of stable TLR9-ODN nucleoprotein complexes is a highly species- and sequence-dependent process (**Figure 3.5**). TLR9 clearly discriminates not only the CpG dinucleotide but also the species-specific higher-order CpG motif. Thus, the sequence-dependent formation of TLR9-DNA complexes is likely indicative of the specificity of TLR9 activation *in vivo*. Indeed, cell proliferations experiments lend support to the hypothesis that sequence-independent functional influence of nucleic acids on formation of active TLR9 complexes is physiologically relevant (**Figure 3.6**). The higher-order regulatory mechanisms reported for TLR9 activation are consistent with the recent observations that Toll regulation involves both receptor-ligand and receptor-receptor interactions (Weber 2005, Gay 2006).

### 4.2 A Model of TLR9 Activation

Receptor dimerization prior to ligand binding is a recurring theme amongst the members of the TLR family, as well as Toll, and is likely conserved in TLR9 (Gangloff 2004, Weber 2005). Recently, the activation of Toll has been proposed to proceed

through a two step mechanism involving the formation of a ligand-free inactive Toll dimer (Weber 2005). The authors have speculated that a similar mechanism might influence the activation of TLR family members.

It is proposed that the sequence-independent cooperative influence of nucleic acids on TLR9 ligand binding is likely mediated through the promotion of TLR9 dimerization to a sensitized ligand-free complex that is primed for binding of activating or non-activating nucleic acid sequences. The cooperative effect exerted by nucleic acids is independent of their incorporation into the sensitized dimeric TLR9 complex. In contrast to the negative cooperative mechanism reported for Toll by Weber, the mechanism of TLR9 activation is proposed to proceed through positive cooperativity. As a result, TLR9 is sensitized to form nucleoprotein complexes with plasmid molecules in the presence of ODNs and conversely TLR9 is sensitized to bind ODNs when in the presence in plasmids. This is likely mediated through the initial formation of ligand-free TLR9 dimers.

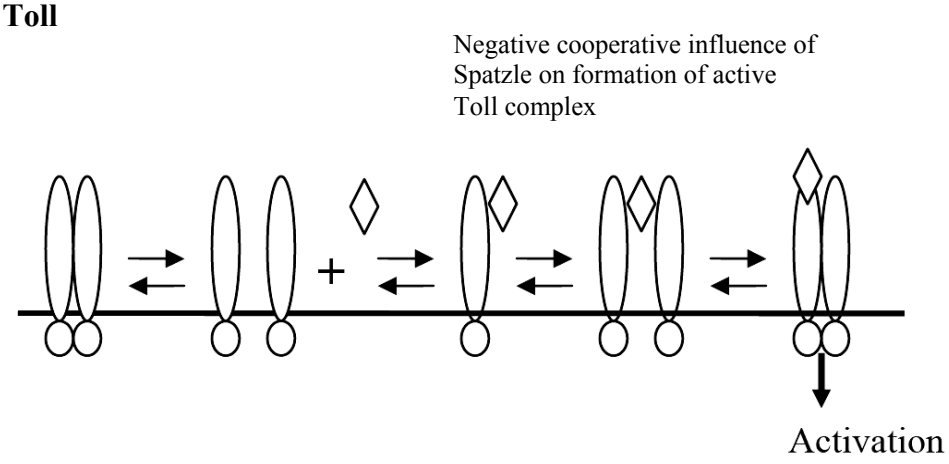
Non-activating nucleic acid sequences, promote the formation of a sensitized, non-active TLR9 dimeric complex, but are rapidly released by the receptor. Indeed, the functional influence of non-activating nucleic acids on the formation of a higher order TLR9 complex does not require integration of these sensitizing nucleic acids into the complex. Labeled ODNs, although able to influence the binding of plasmid by TLR9, were not incorporated into TLR9-plasmid DNA complexes.

Following release of non-activating sequences, the ligand-free TLR9 dimer persists for a short period of time. Although this dimer is inactive, it has been 'sensitized' and therefore has increased ligand affinity. Thus, the sensitized inactive TLR9 dimer is primed for ligand 'sampling'. The binding of activating sequences by the

ligand-free dimer would be hypothesized to induce structural alterations within the TIR domains of the TLR9 dimer leading to receptor activation. In contrast to the Toll model, the conformational changes induced within TLR9 following the sequence-independent interaction with nucleic acids, increase the affinity of the receptor for the stable binding of activating or non-activating sequences. This results in the second step of TLR9 activation proceeding with a much higher affinity than the first step. The equilibrium between TLR9 monomers, ligand-bound dimers, and ligand-free TLR9 dimers is illustrated in **Figure 4.1**. Supportive of this model of higher order functional influence of nucleic acids on TLR9 activation, the sequence-independent association of DNA with TLR9 has become increasingly well established (Latz 2004, Yasuda 2006).

Collectively, the results presented here demonstrate that nucleic acids of activating and non-activating sequences are able to influence the activation of TLR9. These influences are of both sequence-dependent and sequence-independent nature and shed light on the wide-range of nucleic acid ligands recognized by the receptor. Previous investigations have lacked consensus regarding the sequence-dependency of ligand binding by TLR9. Receptor activation clearly demonstrates a preference for nucleic acids of activating sequence *in vivo* whereas TLR9 has demonstrated sequence-independent interactions with nucleic acids *in vitro*. This investigation adds clarity to these discrepancies. The activation of TLR9 is highly-sequence dependent; however, non-activating nucleic acids are able to modulate TLR9 responsiveness through receptor sensitization. Based on these observations and the homology between the Toll and TLR receptor families, a two-step model describing TLR9 activation is proposed. The initial binding event of this model determines receptor specificity and the second binding event

**A) Toll**




**B) Toll-Like Receptor 9**

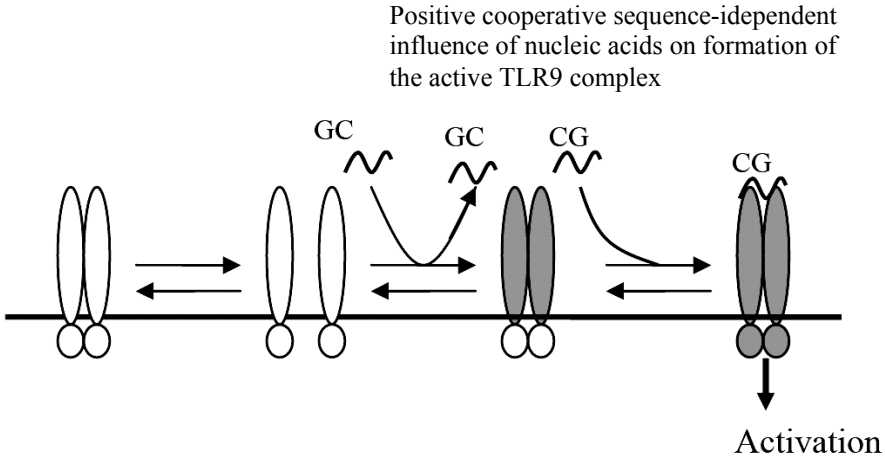



**Figure 4.1: Models of Activation of Toll and Toll-like Receptor 9. A) Toll.** Activation of Toll is mediated through a two-step reaction. The first binding event, to the formation of a ligand-bound monomer, occurs with high affinity. The second stage of Toll activation, binding of the Toll-ligand monomer by a ligand-free monomer, occurs with lower affinity, representing negative cooperativity of activation. **B) Toll-like Receptor 9.** Activation of TLR9 is proposed to follow a similar two-step reaction with the distinction being that the second step to dimer formation occurs with positive cooperativity.

dictates receptor sensitivity, in a positive or negative manner. This model has physiological significance as the sequence-independent cooperative effect of nucleic acids on TLR9 may be in the determination of the activation set point for the receptor. That increased concentrations of nucleic acids lead to system sensitization, in a sequence-independent fashion, suggests that the release of bacterial nucleic acids in the phagosome following cell lysis serve to prime the TLR9 system. Thus, activating CpG motifs within microbial DNA will activate the receptor in an expedient fashion for the generation of innate immune responses. This dual requirement for TLR9 activation may also prevent the potentially deleterious activation of the system as the primed TLR9 dimer is also able to be inactivated by inhibitory nucleic acid sequences found within host DNA and therefore serves as a safeguard function.

### 4.3 Host Defense Peptides as Activators of the PhoPQ Two-Component Sensory System

In a manner analogous to that of Toll-like Receptor 9 of the vertebrate host, the two-component systems of bacteria act as sensory systems that monitor the extracellular environments and initiate responses for increasing microbe survival. These systems have undergone extensive modifications throughout millions of years of evolution for maximizing evasion and adaptation strategies (Peschel 2006). As the TLR family has evolved to identify and respond to a broad-range of conserved microbial biomolecules, the PhoPQ two-component system has evolved to recognize conserved host molecules. These responses culminate in the activation of increased microbial virulence phenotypes. Although the traditional ligand for the PhoPQ system has been regarded as extracellular

$Mg^{2+}$ concentrations, host defense peptides have recently been observed to activate PhoPQ.

Host defense peptides are an essential component of innate immunity in both higher and lower organisms. Recently, considerable interest has been generated in regards to employing HDPs as templates for the rational design of novel antimicrobials. In the face of an increased propensity of multi-drug resistant bacterial strains, HDPs may offer an alternative to conventional therapies. Although the use of an evolutionarily refined antimicrobial molecule seems logical, this must be tempered with an appreciation for these molecules to promote resistance phenotypes. As HDPs serve as an essential component of the innate immune arsenal, the consequences of microbial HDP resistance could be profound. Indeed, sub-lethal HDP concentrations, as are often employed in immunomodulatory therapeutic applications of HDPs, have been demonstrated to exert a selective pressure on the development of resistant phenotypes (Zhang 2005, Nizet 2006). Thus, the development of novel HDP therapeutics must also consider the ability of these molecules to activate bacterial defensive responses.

Traditionally, host defense peptides have been regarded as bi-functional molecules with direct antimicrobial activity and the ability to modulate innate and adaptive immune function (Brown 2006). This investigation proposes a third activity for HDPs: the activation of bacterial defensive responses and virulence characteristics by way of PhoPQ. Thus, the selection of HDPs based on their inability to initiate PhoQ activation would provide a rational basis for HDP evaluation. To this end a mathematical model is proposed that discriminates and quantifies HDPs based on direct antimicrobial activity and PhoQ activation potential. This model is based upon MIC values against *S. typhimurium* wild-type and PhoPQ mutant strains.

Analysis of a subset of peptide derivatives based on Bac2A, a derivative of the bactenecin peptide, demonstrated that the antimicrobial efficiency against either of the PhoP- or PhoQ- mutants fell within a limited range of values (**Table 3.1**). This suggests that most single amino acid substitutions do not impact the direct antimicrobial activity of host defense peptides. A notable exception peptide P7, with proline at position 7 of the peptide, has vastly diminished direct antimicrobial activity as compared to other members of the peptide set. This demonstrates the potential for amino acid substitutions to affect peptide activities likely resulting from the distortion of secondary structure. The primary difference amongst the members of the peptide subset was the ability to activate bacterial defensive responses through the activation of PhoPQ. Based on the results presented, peptides that function as the least effective PhoQ ligands tend to be the most effective antimicrobials. Presumably, this likely results from a lack of PhoQ-dependent bacterial adaptation as peptides with weak PhoQ ligand activity will encounter bacterial membranes devoid of HDP resistance characteristics. Notably, the difference in magnitude of PhoQ activation amongst the peptide derivatives is comparable with the levels of resitance to cationic peptides resulting from the activation of PhoPQ by low magnesium concentrations (MacFarlane 2000).

Based on the results of the quantitative assay system the ability of HDPs to evade PhoQ detection was found to be the most significant factor in determining antimicrobial activity (**Table 3.2, Figure 3.8**). The physiological relevance of these observations is supported by the demonstrations that peptides calculated to elicit the greatest bacterial defensive response coincided with those found to initiate the highest levels of PhoQ activation in the *Salmonella* reporter strain. Peptide K7, having the greatest BDR score amongst the peptide derivatives, initiated the greatest induction of PhoQ in the

*Salmonella* reporter strain (**Figure 3.9**). Conversely, Sub3 and R3 initiated the lowest amount of PhoQ activation in the reporter strain and also had the lowest calculated BDR scores.

It is also of note that the magnitude of PhoQ activation induced by K7 in the reporter strain was comparable to that of micromolar magnesium, a highly potent environmental PhoPQ stimulus. This is supportive of concerns that HDP therapeutic developments not accounting for the ability of these peptides to initiate bacterial defensive responses could induce the development of HDP-resistant bacterial phenotypes. As a testament to the ability of sub-lethal concentrations of HDPs to activate PhoPQ, and thus increase bacterial HDP resistance, pre-exposure of bacteria to HDPs results in increased resistance to a spectrum of HDPs. This demonstrates the validity of the quantitative assay system as peptides predicted to be the strongest activators of PhoQ, based on their high BDR values, also activate the strongest adaptive resistance phenotypes *in vivo* following bacterial pre-exposure. It is of note that the increases in bacterial resistance were of far lower magnitude than those initiated by extracellular $Mg^{2+}$. This can be explained by two phenomena. Firstly, the treatment of bacteria with sub-MIC concentrations of HDPs will invariably result in the induction of bacterial defensive responses by way of the PhoPQ TCS. The activation of PhoPQ results in the induction of the protease PgtE, resulting in the reduction of the effective concentration of sub-MIC HDP during the incubation period. This is in contrast to $Mg^{2+}$, which does not suffer from these same stability concerns. Secondly, the treatment of *Salmonella* with HDPs results in the induction of a subset of the PhoP-regulated genes (Bader 2003). This contrasts with the micromolar $Mg^{2+}$ concentrations, which induces the entire PhoP-regulon (Bader 2003). Thus, the treatment of bacteria with sub-

MIC HDPs may result in only partial modification of the *Salmonella* cell membrane. This must however be tempered with the appreciation that even minor changes to this entity can have drastic consequences on bacterial susceptibility to therapeutic treatments with HDPs. These results perhaps foreshadow the potential for HDPs to induce HDP resistance bacterial phenotypes and offer a mathematical assay system for prediction of these peptide tendencies.

The quantitative HDP system presented here provides a tool for the rapid analysis of direct antimicrobial bacterial defensive response initiation activities. While it can be argued that the PhoPQ system is not the sole mechanism of HDP resistance within microbes, it is a system central to such pathogenic responses in a broad spectrum of bacteria. In addition, the crystallographic structure of the PhoQ sensory domain enables the rational design of peptides that have increased PhoQ evasiveness. This system provides a less costly and more rapid screening procedure for HDPs than the current basis thus allowing for a more focused approach to therapeutic HDP design.

### 4.3 A Model of PhoQ Peptide Binding

The determination of a crystallographic structure of the *S. typhimurium* PhoQ sensory domain, in combination with a predicted host defense peptide binding region, has afforded the opportunity to form hypotheses regarding the interaction of HDPs with PhoQ. The model presented by Bader predicts that a large acidic region running planar to the bacterial cell membrane represents the peptide binding region (Bader 2005). According to the authors, the coordination of divalent cations between the negatively charged bacterial membrane and the acidic surface of PhoQ removes the electrostatic

repulsion between these two electronegative groups and locks the periplasmic domain of PhoQ to the membrane in an inactive form. In a low divalent cation environment, it is predicted that the removal of the stabilizing cation bridges results in electrostatic repulsion between PhoQ and the cell membrane with displacement of PhoQ from the membrane and formation of an active conformation.

The membrane-associated, ligand-binding region of PhoQ is defined by a regularly repeating pattern of acidic side chains contributed by two anti-parallel strands comprised of residues 112-125 and 152-167. It is predicted that residues Asp152, Glu154, Glu123, and Glu121 provide a regular pattern of carboxyl-group containing side chains that contribute to HDP binding. This region was demonstrated to responsible for divalent cation coordination by Bader through a variety of experimental techniques and calcium ions included in the crystallization conditions were found to be coordinated to the same region (Bader 2005).

The ability of host defense peptides to activate PhoQ is dependent upon the ability to provide suitable electrostatic interactions to replace those provided by the cations. It is postulated that the ability of peptides to form electrostatic interactions with PhoQ would be best achieved when the peptides are in alpha-helical conformation. Circular dichroism investigations of linear derivatives of the peptide bactenecin indicate that the peptide assumes random conformation in solution but forms alpha-helices in hydrophobic environments (Wu 1999). These peptides model as amphipathic, alpha-helix with one face of the helix dominated by positively charged residues including Arg1, Arg4 and Arg12. Based on the structural properties of an alpha-helix, it is predicted that these positively charged groups would be in close accordance with the

negatively charged residues in PhoQ thus permitting the displacement of calcium ions (**Figure 4.2**). This displacement would culminate in PhoQ activation and may represent a conserved feature for host defense peptides that adopt amphipathic alpha-helical conformations.

### 4.4 Host Defense Peptides and Retro-Inversion

Host defense peptides appear a logical alternative to conventional antibiotics. HDPs have demonstrated direct antimicrobial activity against a diverse spectrum of bacteria, modulation of innate immune responses, and regulation of inflammatory processes (Finlay 2004). Although these represent promising attributes for the large-scale production and therapeutic application of HDPs, issues relating to peptide manufacturing costs have limited applications to topical treatments such as polymyxin B and gramicidin S for surface infections. Thus far, the predominant strategies for the reduction of associated production costs have focused on measures to increase peptide production outputs, minimization of peptide size, and the integration of stabilizing modifications. The use of stabilizing modifications in HPDs include all D-amino acid peptides, non-standard peptide backbones such as peptidomimetics, liposome formulations, and chemical modifications that bestow protease resistance.

Peptide antibiotic developments that incorporate non-standard amino acids and linkages have used nature as a template; non-ribosomal peptide synthetase complexes are often employed by microbes to produce peptide-based antibiotics. These alternative peptide strategies often use D-amino acids, non-standard amino acids and unique

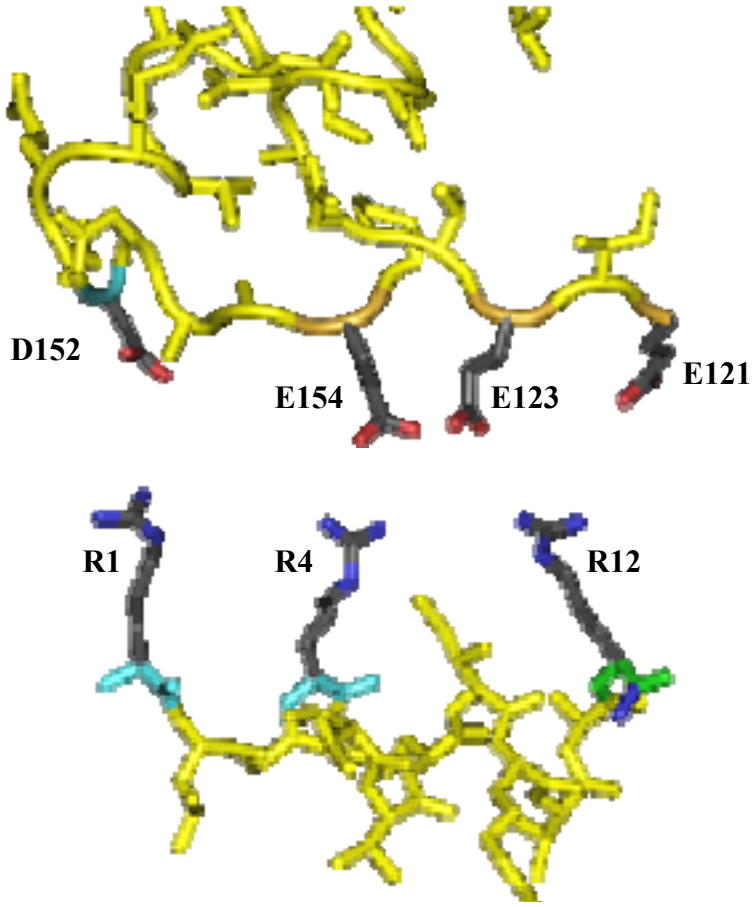



**Figure 4.2: Molecular Structure of the Salmonella PhoQ Sensory Domain and its Interaction with Bac2A.** A model of the metal binding region of the sensory domain demonstrating how alpha helical cationic antimicrobial peptides could displace bound cations leading to activation of the protein. Structures were generated with Cn3D version 4.1.

patterns of modification to produce molecules with enhanced evasion of bacterial defensive and adaptive strategies. Host defense peptide development may also draw upon the therapeutic applications of TLR9 agonists and antagonists. Indeed, the use of phosphothioate-modified ODNs as novel therapeutics has considerable advantages over their natural counterparts due to increased stability against nuclease degradation and similar manufacturing costs. As such, PTO-ODNs have emerged as the standard for both the investigations and clinical applications of TLR9 agonists and antagonists. A similar strategy for HDP therapeutic development would drastically reduce the long-term costs associated with natural HDP applications as increased stability would decrease the need for repeat applications.

Retro-inversion as a means of increasing host defense peptide stability and conserving biological activity would seem a likely alternative for therapeutic HDP development. RI-peptides have reversed amino-acid sequences and incorporation of D-amino acids. Thus, the RI-modification results in maintenance of three-dimensional structure and identical placement of side chain residues, thus conserving biological structure/function relationships. Importantly though, the reversal of peptide bonds within these molecules renders the peptide resistant to the action of protease enzymes.

To examine the application of retro-inversion to host defense peptides, BMAP-28, a bovine host defense peptide with demonstrated therapeutic potential was chosen. BMAP-28 is active at micromolar concentrations against a broad range of Gram-positive and Gram-negative microbes, including multi-drug resistant strains, and importantly these antimicrobial activities are retained in the presence of physiological salt concentrations. Animal challenge models have also demonstrated that BMAP-28 protects mice from lethal i.p. infections (Cirioni 2006) and reduces lethality against

*Staphylococcal* species at concentrations below the host toxic threshold (Giacometti 2004). Although the immunomodulatory activities of this peptide have not been studied in detail, BMAP-27, another bovine cathelicidin, has demonstrated immunomodulatory activities (Mookherjee 2006).

This investigation has demonstrated that the retro-inverso form of BMAP-28 has maintained or improved function, as compared to the natural peptide, and significantly increased resistance to proteolytic degradation. The expectation has been that systematic inversion of the stereochemistry at the backbone alpha-carbons, in combination with reversed amino acid sequence, would yield proteolytically stable retro-inverso peptide isomers. As a direct antimicrobial agent, RI-BMAP28 was 2-3 fold more potent than the natural isomer against a broad range of Gram-negative bacteria (**Table 3.3**). That RI-BMAP28 is vastly superior to the natural isomer in terms of proteolytic stability, and protease secretion is a common theme amongst bacteria as a defensive response, suggests that this increase in direct antimicrobial activity is likely due to increased stability as opposed to enhanced activity (**Figure 3.11**). Supporting this hypothesis, the RI-peptide had comparable increases in antimicrobial activity against all of the bacterial strains tested. If the RI-modification indeed enhances antimicrobial activity it would be expected that a more diverse range of MICs would be found between the peptide isomers against different bacterial strains. In direct contrast to the increased antimicrobial activity of the retro-inverso isomer, peptide cytotoxic activity was drastically reduced upon retro-inversion of BMAP28 (**Figure 3.13**). The administration of the natural peptide resulted in nearly complete cell lysis at the highest concentration tested whereas RI-BMAP28 demonstrated no lytic activity. Indeed, it is enticing to speculate that retro-inversion may in fact decrease cytoxicity concerns regarding HDP

administrations. This must however be investigated with a broad-range of HDPs to see if it is a recurrent phenomenon amongst RI-modified HDPs. Although no definitive conclusion regarding the nature of this phenomenon can be made it is speculated that the cytotoxic activity of BMAP28 is mediated through a specific interaction with a host cell component. Indeed, the human cathelicidin LL-37 has been observed to modulate apoptotic pathways in human primary innate-immune effector cells and was proposed by the authors as an innate defense response to infected cells (Barlow 2006). That the RI-isomer results in decreased cytoxicity suggests that this interaction is sensitive to the change in hydrogen bonding pattern presented by the main chain of the peptide and may be a particularly desirable trait if the other non-host cell activities of the modified peptide are maintained. Protein-peptide recognition is dominated by hydrophobic and electrostatic interactions where the latter, particularly dominated by hydrogen bonds, can involve the peptide backbone and side chains of polar and charged residues. Upon RI-modification, the amide bonds of a peptide are inverted with respect to the native ligand and may account for the observations that retro-inversion is not universally successful in mimicking native peptides as the result of decreased affinity for the receptor binding site (Fischer 2003).

The results presented here lend support for the efficacy of HDPs, and in particular those that have undergone RI-modification, for therapeutic applications. Retro-inverso modification of BMAP28 results in conserved immunomodulatory activities to that of the natural peptide, on the basis of suppression of LPS-induced TNF-α secretion (**Figure 3.14**). In contrast to BMAP28 however, RI-BMA28 had improved proteolytic stability and antimicrobial activity with decreased cytotoxic activity. As RI-BMAP28 appears to meet the standards for safety and efficacy of HDPs as therapeutics,

with improved biological stability, RI peptide modification represents a potential mechanism for decreasing the prohibitive cost of HDP therapies due to a hypothesized reduction in dose frequency.

Thus, the analyses presented here lend support for the manipulation of natural ligands of both prokaryotic and eukaryotic sensory systems in the development of novel therapeutics. An increased understanding of the complexity of the interactions of these novel ligands with their biological targets will allow for a more calculated approach in therapeutic developments that offers minimal toxicity to the host while maximizing the biological activity of the ligand.

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