Activation and Regulation of Toll-like Receptor 9: CpGs and Beyond

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Abstract: Toll-like receptor 9 has been the focus of considerable research attention for the ability to modulate its activity, and subsequent innate immune responses, through DNA-based immunotherapeutics. Nucleic acids are attractive as therapeutics for their low cost, chemical stability and ease of production. While the ability for TLR9 to be differentially regulated by nucleic acids of varying sequences and structures offers flexibility for immunotherapeutic design, it also necessitates a more comprehensive characterization of these agonists in terms of how these structural parameters correlate with the activation of unique cellular responses. Despite the utilization of TLR9 agonists in human trials these issues have not been adequately addressed. While a wealth of cell stimulation experiments demonstrate the preferential ability for nucleic acids which contain unmethylated cytosine-phosphate-guanine (CpG) motifs to initiate innate immune responses this has not been supported by binding investigations from which largely contradictory information has emerged with respect to the ability of TLR9 to bind nucleic acids in a sequence-dependent fashion. Recent models help to reconcile this apparent contradiction by suggesting that while TLR9 activation is specific for CpG-containing nucleic acids, the receptor binds, and is functionally influenced by, nucleic acids in a sequence-independent fashion. We have proposed a model in which the absolute concentration of nucleic acids modulates the sensitivity of the receptor in a sequence-dependent fashion while activation is specifically achieved by CpG-containing ligands. In this review we reconsider the literature from the perspective of this new appreciation of the functional complexity of TLR9 ligand binding and higher-order regulation with discussion of the implications for immunotherapeutic targeting of TLR9.

1. INTRODUCTION

The mammalian immune system consists of innate and adaptive responses [1]. Adaptive immunity is mediated largely through the production of antibodies and T cell responses which are specific to, and offer protection from, previously encountered immunological challenges. The establishment of adaptive immunity occurs over the course of days to weeks. In contrast, innate immune responses, which are activated within minutes, represent a non-specific defense mechanism to limit the early proliferation and spread of infectious organisms [2]. Despite initial characterizations to the contrary, the innate immune system has considerable specificity, in particular in its ability to discriminate pathogens from self. Specific and appropriate engagement of the innate immune system is achieved by targeting molecules, termed pathogen-associated molecular patterns (PAMPs), which are highly conserved in microbes but largely absent from the host cell anthology of biomolecules. Recognition and response to these PAMPs is facilitated through pattern recognition receptors (PRRs) that include the Toll-like receptors (TLRs). The TLRs, upon recognition of their cognate ligands, mediate activation of innate immune responses [3].

There is a growing appreciation that modulation of innate immune responses may represent an effective strategy to bolster host immunological defenses and the ability of TLRs to initiate these responses makes these receptors logical immunotherapeutic targets [4]. This enthusiasm is tempered however by the recognition that excessive activation of innate immune responses can result in considerable harm to the host, including rheumatoid arthritis [5] and endotoxic shock [6]. These considerations emphasize the importance of better understanding the structure/function relationship of the TLR agonists, as well as developing a greater appreciation of the emerging complexity of Toll-like signaling.

1.1. Toll

The "Toll-like" nomenclature reflects similarity to Toll, a *Drosophila* receptor with involvement in the establishment of dorsoventral polarity during embryogenesis [7], and activation of innate immune responses in the adult fly [8]. The involvement of Toll in mediating innate immune responses and the discovery of sequence homology between the cytoplasmic domains of Toll and the human interleukin receptor (IL-1R) eventually led to the discovery of Toll-like receptors in vertebrates [9, 10]. In addition to functioning as a stimulus for the discovery of the Toll-like receptors, Toll remains an effective model to elucidate some of the more complex functions of the Toll-like family.

1.2. Toll-Like Receptors

The Toll-like family is highly conserved in vertebrates, including 11 human homologues that have been described to date. Each member is activated by a unique ligand, or in some cases, a unique class of ligands. In general, activation of the Toll-like system initiates both direct anti-microbial mechanisms, such as the stimulation of pro-inflammatory

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cytokines [11, 12], as well as prompting the activation of the adaptive immune responses [2]. Classically, signaling through the Toll-like systems involves an intracellular cascade involving myeloid differentiation primary response gene 88 (MyD88), interleukin-1 receptor activated kinase (IRAK) and tumor-necrosis factor receptor-associated factor 6 (TRAF6) leading to the activation of NF-κB [13, 14] (Fig. **1**). While this represents an accurate baseline description of the cellular mechanism of the Toll-like family it is becoming increasingly apparent that these systems are far more complex than initially hypothesized in terms of both ligand recognition and utilization of alternate signaling pathways [15].

1.2.1. TLR Structures

TLRs are highly conserved type I integral membrane proteins. Intracellularly, TLRs are similar to the interleukin-1 receptors on the basis of a highly conserved Toll-IL-1 receptor (TIR) domain [16]. The ligand-binding domains of the Toll-like receptors are situated either on the cell surface or in intracellular compartments. The intracellular Toll-like receptors include TLR3, 7, 8 and 9 which in addition to their cellular location, also shares the common characteristic of being activated by nucleic acid PAMPS. In contrast to other TLRs, such as TLR4 and TLR5, which recognize lipopolysaccharide and bacterial flagellin respectively, the nucleic acid binding TLRs are unique in that they are activated by PAMPs which closely resemble endogenous molecules. They are therefore at greater risk for activation by self molecules and their sequestration within intracellular locals may reflect a strategy to avoid this occurrence.

For all TLRs, the ligand binding domains consist primarily of a repeating pattern of a leucine-rich repeat (LRR) motif [17]. This motif is present in a large number of eukaryotic proteins, while of diverse function, typically share the unifying characteristic of involvement in biomolecular recognition and interaction processes [18]. Indeed the primary function of the LRR motif appears to be in providing an adaptable structural matrix for biomolecular interactions, the versatility of which is exemplified by the vast and structurally diverse ligands recognized by the TLR family.

1.2.2. Activation Through Dimerization

The formation of higher order structures has been demonstrated as a signaling prerequisite for both Toll as well as a number of TLRs and likely represents a conserved mechanism of activation. TLR activation can occur through the formation of hetero-dimers, as is the case for TLR1 and TLR6 [19], while other TLRs, such as TLR3, TLR4 and TLR8, are activated through the formation of homo-dimers [20]. Formation of dimers is not sufficient to result in activation of all TLRs, as demonstrated by the inability of antibody-induced dimerization to result in activation of all members of this family [21, 22]. This may reflect the additional prerequisite for activating structural alterations that are induced specifically upon the binding of appropriate ligands. The existence of structural and organizational variations of the TLRs, including monomers, inactive dimers and active dimers, raises the potential for regulation of signaling activity *via* modulation of the equilibrium between the different forms of the receptor.
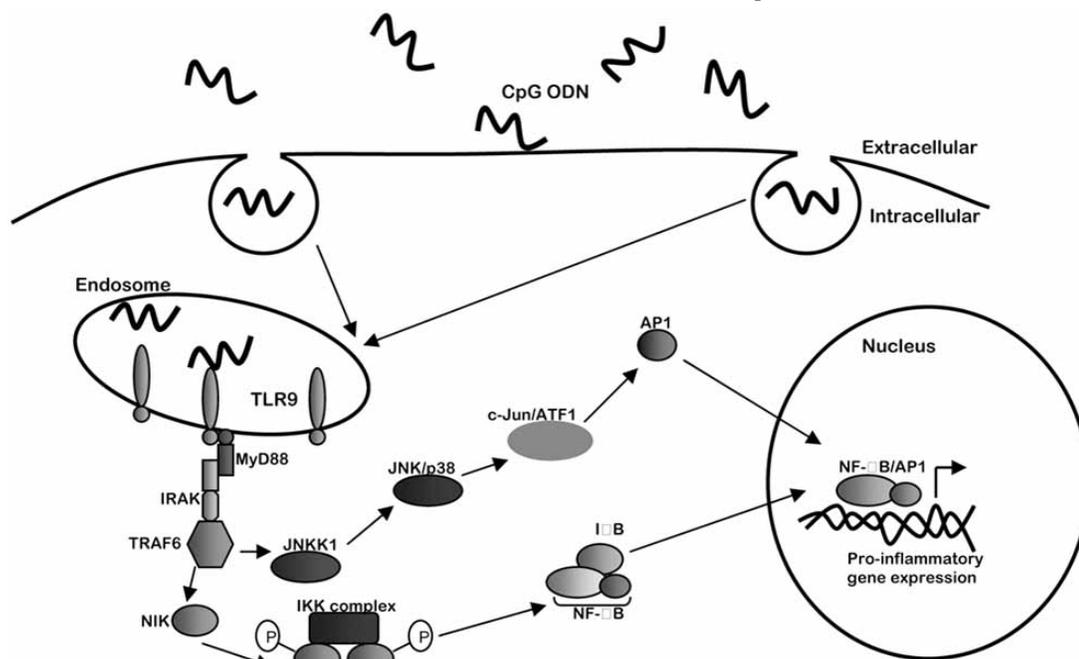
Fig. (1). Signal Transduction Associated with Activation of Toll-like Receptor 9. Binding of bacterial DNA or CpG ODNs promotes activation of TLR9 through dimerization. The ligand-bound dimer recruits MyD88 to the membrane and initiates association with TOLLIP and IRAK4. MyD88 activates IRAK4 to phosphorylate 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 immunomodulator effectsincluding cell-mediated immunity, apoptosis as well as direct antimicrobial activity.

1.2.3. Pathogen-Associated Molecular Patterns

One of the more remarkable properties of the Toll-like family is its ability to recognize a vast and structurally diverse spectrum of ligands [23]. Indeed the ability of this family of germline-encoded, non-clonal receptors to be specifically activated by such a broad range of chemically-diverse ligands has been described as one of the most intriguing and important questions of Toll-like functioning [24]. Some of the identified TLR ligands are presented in (Table **1**). Currently there is a scarcity of information available regarding the structural mechanisms of ligand binding by the TLRs. This, along with the complexity of the structure/function relationship of ligand-binding, has limited the rational design of TLR agonists and antagonists.

1.3. Toll-Like Receptor 9 and its Ligands

There is considerable evidence that microbial DNA serves as the physiological ligand for TLR9. Most convincingly, TLR9 knockout mice are unresponsive to bacterial DNA but are rendered responsive through TLR9 expression [25]. Foreign and host DNA appear to be discriminated by the receptor on the basis of unmethylated cytosine-phosphate-guanine (CpG) dinucleotide sequences which are frequent within microbial DNA but largely absent from host cell genetic material [26, 27]. Activation of TLR9 also appears to involve recognition of sequence elements above and beyond the CpG dinucleotide as TLR9s from different species are preferentially activated by unique, species-specific, higher-order motifs of six bases in length. For example, mouse

TLR9 is most responsive when the CpG motif is flanked by purine residues on the 5' side and pyrimidine residues on the 3' side, in particular GACGTT [28-30]. In contrast, human TLR9 has the greatest degree of responsiveness to GTCGTT or TTCGTT sequences [30]. Following the expression of either mouse or human TLR9 in TLR9⁻ cells, the cells are rendered responsive to ODNs in a species-specific fashion, i.e. expression of mouse TLR9 results in preferential responses to ODNs that contain the preferred mouse sequence motif [31]. This suggests that sequence-specific cellular activation reflects preferential binding by TLR9.

1.3.1. Localization of TLR9 to Limit Activation by Self DNA

That the ligand for TLR9 so closely resembles an endogenous molecule raises the potential for the inappropriate activation of innate immune responses through the binding of self DNA. There appear to be cellular mechanisms in place, above and beyond the requirement for unmethylated CpG motifs, to limit activation of TLR9 by self DNA. One such determinant of ligand specificity may be the intracellular localization of TLR9. The localization of host DNA within the nucleus of normal, healthy cells sequesters these potential ligands from TLR9 thereby muting their activating potential. The induction of innate immune responses by host DNA also appears to be limited by inhibitory sequences that are present within the telomeric regions of mammalian DNA [32]. These inhibitory sequences likely function to counter any activation that is initiated by the association of TLR9 with self DNA, i.e. self DNA is inherently inhibitory to TLR9.

Table 1.   Toll-like Receptors and their Ligands

Receptor	Ligand
TLR1	Triacyl Lipopeptides
TLR1/TLR2	Triacyl Lipopeptides Lipoarabinomannan (LAM) from mycobacterium, Yeast/ Zymosan, Glycosylphosphatidyl Inositol-Linked Proteins
TLR2	Lipoprotein, Peptidoglycan, Zymosan
TLR2/TLR6	Diacyl Lipopeptides, Lipoteichoic Acid, Yeast/ Zymosan, Glycosylphosphatidyl Inositol-Linked Proteins
TLR3	Double-stranded RNA
TLR4	Lipopolysaccharide
TLR5	Flagellin
TLR6	Diacyl Lipopeptides, Lipoteichoic Acid, Zymosan
TLR7	Single-stranded RNA, Imiquimod/Resiquimod
TLR8	Single-stranded RNA
TLR9	Non-methylated CpG- Containing DNA
TLR10	Unknown
TLR11	Urogenic bacteria
TLR12	Unknown
TLR13	Unknown
TLR15	Unknown

In the resting state, TLR9 is situated within the endoplasmic reticulum and upon stimulation undergoes rapid translocation to the endosome [33]. Utilizing a hybrid TLR9 that is localized to the cell surface Barton *et al.* demonstrated that this relocated TLR9 responded normally to ODN ligands but was also able to respond to self DNA, which is not observed for endogenous TLR9 [34]. Similarly it has been demonstrated that endosomally translocated self DNA can activate TLR9 while extracellular self DNA cannot [35]. This suggests either specificity in the uptake of nucleic acids, with selective endocytosis potentially serving to limit the exposure of TLR9 to inappropriate ligands, or that self DNA is usually degraded before it has the opportunity to function as a TLR9 ligand. Interestingly, Lande *et al.* recently demonstrated that an integral component of the innate immune system, the host defense peptides, may facilitate the endosomal translocation of self-DNA. Indeed, the human cathelicidin LL-37 was found to bind host DNA and activate the innate immune system in a TLR9-dependent manner and may therefore, in conditions of HDP overexpression, provide a mechanism for the breaking of tolerance to host molecules [36].

The role of ligand aggregation and multimerization in the activation of TLR9 by nucleic acid has also been investigated. Wu *et al.* have demonstrated that the activation of TLR9 by natural (phosphodiester) DNA was not merely a function of the presence of a CpG motif but also influenced by the ability of these molecules to form aggregates and rigid secondary structures within the central CpG motifs [37]. Indeed, CpG ODNs that were devoid of aggregation were found to act as receptor antagonists thus the design of TLR9-based therapeutics may not only depend on primary structure but also secondary structure characteristics.

While there appears to be mechanisms in place to limit the activation of TLR9 by self DNA, the limited induction of innate immune responses by endogenous molecules may serve a physiological function to initiate localized inflammation in response to damaged or dying cells. Emerging evidence suggests that host DNA has TLR9-activating potential and that presentation of host nucleic acids might allow for induction of advantageous, localized innate immune responses [38-40]. Recently, Ishii and Akira have suggested that TLR9 has evolved to detect not only the CpG motif of pathogenic organisms, but also other molecular patterns that are associated with host-derived DNA, such as CpG derivatives of an as of yet unidentified nature or some other conserved motif [41].

The mechanisms to limit activation of TLR9 by self 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 the conditions under which they physiologically occur. As the late endosome provide a unique environment which is chemically defined by an acidic pH, changes in the protonation state of either the receptor or ligand may be prerequisite for the formation of an activated complex. Secondly, that TLR9 has the ability to be functionally influenced 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.

1.3.2. pH Dependence of TLR9 Ligand

Physiologically, the interaction between the endosomal TLRs and their nucleic acids ligands would be anticipated to be optimized for the acidic pH of the late endosome. This has been verified through *in vitro* investigations of TLR3 and TLR8 which have demonstrated that ligand binding and dimerization are more efficiently mediated at acidic pHs [42, 43]. Similarly, CpG-DNA-driven signaling *via* TLR9 requires acidification and maturation of endosomes and is effectively blocked by agents, such as chloroquine or bafilomycin A1, that interfere with either endosomal trafficking or acidification [44]. Surface plasmon resonance investigations of TLR9 suggest that this pH-dependence is mediated at the level of ligand binding as optimal interaction between TLR9 and CpGs is observed at acidic pH [45].

There are two potential, although not mutually exclusive, mechanisms that may account for the pH-dependent activation of TLR9 by nucleic acids. Firstly, the ionization state of the TLR9 ectodomain may be a critical determinant of the ability of the receptor to either bind its ligand or to mediate dimerization to an active complex. Notably the ectodomain of TLR9 contains several histidine residues, one of which is also conserved in TLR7 and TLR3. As the pKa for the imidazole ring of histidine is close to physiological pH these residues are among the most pH-sensitive components of a protein. A conceptually similar, but functionally inverted, mechanism to regulate ligand binding and release 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 and during endosomal acidification changes in the protonation state of a pseudoligand of the ectodomain serving to displace the true ligand [46].

Alternatively, changes in the protonation state of the ligand may dictate its ability to be bound by the receptor. The ionization of nucleic acids, and in particular cytosine residues, may change during the maturation of the endosomes. While the pKa for cytosine in 4.2 this value is likely considerably higher in the context of a nucleic acid strand as the polyanionic environment would stabilize positively charged groups. As such, a significant fraction of these residues are likely positively charged within the endosome and this may represent a critical determinant for binding by TLR9. Based on these observations, there may be potential 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 [43].

1.3.3. Oligodeoxynucleotides (ODNs)

The immunostimulatory action of bacterial DNA can be effectively mimicked with synthetic, single-stranded oligodeoxynucleotides (ODNs) that are typically 24 to 30 nucleotides in length. As therapeutic TLR9 agonists these ODNs have considerable advantage over bacterial DNA as a result of their defined sequence/structures, their low cost of

synthesis and chemical stability. To achieve greater biological stability through nuclease resistance, the majority of TLR9 investigations utilize phosphothioate-modified (PTO) ODNs in which one of the non-bridging backbone oxygen atoms of the phosphodiester linkage is replaced with sulfur.

1.3.4. Classes of ODNs

Three classes of ODNs, which differ in their sequences and in the presence and location of backbone modifications, have been defined on the basis of their ability to elicit unique cellular responses. For example, A-Class CpG ODNs (CpG-A) are especially potent at inducing interferon (IFN) α production by plasmacytoid dendritic cells (pDCs). Interferon production leads to the subsequent activation of natural killer (NK) cells and IFN-γ secretion [47]. B-Class CpG ODNs (CpG-B) are potent B cell activators resulting in increased Major Histocompatibility Class II complex (MHCII) expression, secretion of immunoglobins and B cell proliferation [26]. Finally, C-Class CpG ODNs (CpG-C), induce both A-Class and B-Class signaling effects [48]. There is contradictory evidence to suggest that the classes of CpG ODNs may traffic differently, both in terms of uptake as well as retention within intracellular vesicles, and that these differences may contribute to the differences in each classes' signaling properties [49-51]. Indeed, a recent investigation by Guiducci *et al.* demonstrated that TLR9 activation in pDCs by CpG ODNs was dependent on both the ability of the ODN to form higher order secondary structures and the endosomal location of the ligand [52]. CpG-A, which can multimerize due to G-rich sequences at the 5' and 3' ends of the oligo, were found to localize predominantly to transferrin receptor-positive endosomes while the monomeric CpG-Bs localized to lysosome-associated membrane protein-1-positive endosomes thus correlating with the different stimulatory activities of these ODNs in pDCs. As anticipated, CpG-Cs were equally distributed amongst both types of endosomes. 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 [15]. Microarray analysis by our group suggests that while NF-κB is responsive to TLR9 activation, that this is not the exclusive mechanism of TLR9-mediated signal transduction [unpublished observation]. This is also consistent with the discovery of secondary signaling pathways that have been identified for other Toll-like receptors involving unique adaptors and interferon-regulatory factor (IRF) transcription factors [15].

Structurally, the three ODN classes are unique in the number and positioning of CpG motifs, as well as in the presence and extent of backbone modifications (Table **2**). When these ODN sequences were subject to computational secondary structure prediction analysis, we observed that many of the ODNs have the potential to adopt higher order structures based upon the formation of hairpin structures. We postulate that the observed ODN class-specific differences in immunostimulation may be dictated by the different "fit" between each class of ODN and the TLR9 molecule. For instance, the binding of each class of CpG ODN and the TLR9 molecule may trigger different receptor conformational changes and/or promote recruitment of unique co-signaling molecules or accessory proteins. This theory is currently under investigation.

1.3.5. Structural Determinants of ODN Efficiency

CpG ODNs are structurally complex molecules with numerous regions, including the nitrogenous bases, sugar groups and phosphodiester linkages, which have the potential to influence their ability to serve as TLR9 ligands. Adding to this complexity, higher order secondary structure characteristics of ODNs are also thought to play a role in the binding of ligand to TLR9 [53]. To elucidate the structure/immunological function relationship of TLR9 agonists, investigations have examined how alterations of different regions of these molecules influence their ability to activate

Table 2.   Representative Sequences, Structures and Biological Actions of the Different ODN Classes. Table adapted from Klinman, D. M. (2004) Immunotherapeutic Uses of CpG Oligionucleotides. Nature Reviews 4, 1-10

ODN Type	Example	Structural Characteristics	Associated Activity
A	GGTGCAT*CG*ATGCAGG**GGGG**	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	TCGTCGTT*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

TLR9-dependent responses. A representative CpG element, with formal numbering, is presented for reference in (Fig. **2**).
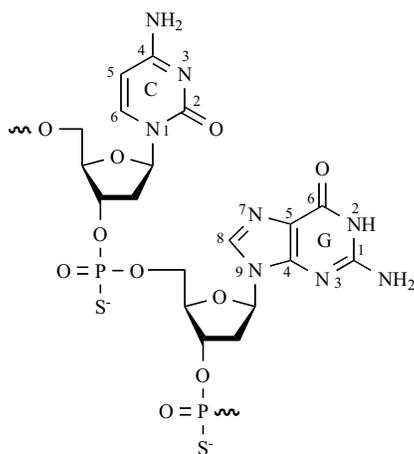
Fig. (2). Chemical Structure of a phosphothioate CpG.

With respect to the nitrogenous bases, a hallmark of CpG activation is that the methylation status of the C5 position of cytosine serves as the primary discriminator between prokaryotic and eukaryotic DNA [25-27]. CpG motifs methylated at this position are rendered ineffective TLR9 ligands in both cell stimulation experiments as well as *in vitro* binding assays. In addition, there are additional positions within the CpG dinucleotide bases that contribute to specificity of interaction with TLR9. A recent investigation by Jurk *et al.* monitored the structure-activity relationship between various cytosine-modified or guanine-modified CpG-ODNs and their subsequent immunostimulatory effects; modification of the hydrogen donor and acceptor function of guanine were investigated in one derivative, C5 modifications of cytosine and N4-alkylated cytosine were investigated in a second derivative [53].

The immunostimulatory activity of CpG ODNs is fairly intolerant to modifications of the core cytosine residue. Replacement of the natural deoxycytidine with either deoxy-5-hydroxycytidine or deoxy-5-methylcytidine reduced the activating potential of the ODNs for human TLR9 by 85% and 70% respectively as compared to the parent CpG ODN [53]. This contrasts however with a report by Kandimalla in which similar modifications of the core cytosine residue in the CpG motif on murine TLR9 were described. Here, replacement of the natural deoxycytidine of the CpG motif with deoxy-5-hydroxycytidine or deoxy-N4-ethylcytine resulted in maintained or improved ability to activate TLR9 [54]. Perhaps a critical distinction between these contradictory reports is that the investigation by Jurk examined responses of human TLR9 while Kandimalla employed a mouse model. Jurk also reported that substitution with deoxy-5-methylisocytidine, deoxyuridine or deoxy-P-base-nuceloside abolished immunostimulatory abilities similar to levels observed for deoxy-5-methylcytidine, the physiologically modified base observed in eukaryotic DNA [53]. Collectively these investigations suggest that for the cytosine residue of CpG dinucleotides it is both the primary exocyclic amino group and the

spatial requirements of C5 that are of fundamental importance for the activity of CpG-ODNs.

Guanine modifications within the CpG dinucleotide of ODNs have also been investigated. The exchange of guanine with 7-deazaguanine resulted in an 81% reduction of human TLR9 activation while substitutions of guanine with 2-aminopurine, purine, 2,6-diaminopurine, or 8-oxoguanine led to reductions in TLR9 activation to 40-60% that of the parent ODN [55]. Replacement of guanine with hypoxanthine maintained the activation potential close to that of the unmodified ODN. Thus, the authors speculated that the guanine base is recognized by the "Hoogsteen base pairing site" of human TLR9 due to the primary recognition factors for the guanine base being N7 and the exocyclic O6. These results, much like those of the cytosine modifications, are in sharp contrast to the previous investigations involving murine TLR9 by Kandimalla [54, 55], particularly in response to the replacement of guanine for 6-thioguanine. The authors rationalized that as the strongest differences between murine and human TLR9 activation were found with O6 replacement of guanine with sulfur, resulting in an isoelectronic derivative, the contrast between murine and human TLR9 may be caused by a spatial constraint effect rather than an electronic effect [55]. Of particular interest was the observation that the deletion of the guanine base in the CpG motif still elicited 30% of the maximal TLR9 activation while deletion of cytosine or replacement with a universal base, such as 3-nitropyrrole or 5-nitroindole, results in complete activation abrogation [55]. Collectively these studies indicate that the cytosine base is of greater importance than the guanine in recognition of the CpG motif and that the 2-keto, 3-imino and 4-amino groups of cytosine and the 1-imino, 2-amino and 6-keto groups of guanine in the CpG motif are important for immunomodulatory activity.

With respect to the sugar groups and their influence on the overall conformation of the dinucleotide, replacement of the deoxynucleosides within the CpG motif with 2'-O-methylribonucleosides abolishes immunostimulatory activity, suggesting that a rigid C3'-endo conformation induced by the 2'-O-methyl modification does not allow proper recognition with TLR9 [56].

The effects of phosphate backbone modifications on CpG ODN immunostimulatory activity have also been investigated. Substitution of an unbridged oxygen for a methyl group on the CpG internucleoside phosphate group results in suppression of immunostimulatory activity, whereas non-ionic phosphate linkages in the flanking sequences can enhance activity [57]. This increase in immunostimulatory activity is dependent upon the location of the modification with respect to the core CpG dinucleotide as substitution with non-ionic internucleoside linkages 5 or 6 nucleotides away in the 5'-flanking region enhance mouse spleen cell proliferation, splenomegaly, and IL-6 and IL-12 production as compared to the parent CpG ODN [57]. The authors suggested that the loss of negative charge at these linkages might allow for tighter interactions between the receptor and modified CpG ODN, with the extent of activation potential independent of the total number of methylphosphonate linkages but rather the positioning of the modified linkage within the ODN [57].

Also supportive of the role of regions outside the CpG dinucleotide, structural alterations of residues contained within the ODN, but outside the CpG dinucleotide, also influence immunostimulatory activity. ODN alterations *via* incorporation of 1',2'-dideoxyribose, or d-spacer, which lacks a nucleobase, alters CpG-ODN immunostimulatory activities in a manner that is largely dependent upon the proximity of the substitution to the CpG motif. Specifically, incorporation of the d-spacer in the 5'-flanking region, 3-5 nucleotides away from the CpG motif leads to enhanced immunostimulatory activity, while those within the 3'-flanking sequence have no effect in comparison to an unmodified parent ODN [58]. Subsequent investigations focusing on the nucleotides surrounding the CpG dinucleotide within the higher-order hexameric sequence demonstrated that both 3'-thymidine residues within the murine GACGTT hexameric CpG motif are critical for TLR9-mediated immune activation [59]. However, substitution of either of the two 5'-nucleobases proximal to the CpG dinucleotide does not result in significant alterations to activity. The most significant influence being determined by the identity of the first nucleotide as the greatest immunostimulation requires a guanosine in the first position with greater variability being tolerated in the residue that is immediately proximal to the CpG motif [59].

Notably all of these investigations of the structure/immunostimulatory function of ODNs are based upon cell stimulation assays rather than observations of direct interaction with TLR9 indicating that some the influences could be mediated through alterations to pharmokinetic parameters such as uptake and/or stability.

1.3.6. Therapeutic Application of ODNs

The low cost, ease of production and stability of DNA ligands make TLR9 an attractive target amongst the Toll-like receptors for immunotherapeutic intervention. The ability for CpG treatment to modulate innate immune responses has given rise to a number of therapeutic applications including: 1) priming the innate immune system to mediate host protection, 2) as adjuvants to promote induction of antigen-specific immune responses, 3) as anti-allergens through establishment of Th1 responses, 4) in the treatment of a variety of malignancies and 5) for improved vaccination efficiency of individuals with poor immune responses [60-64]. There have been over a dozen clinical trials involving more than 500 human subjects examining the immunotherapeutic potential of CpG ODNs.

In spite of the considerable therapeutic potential of CpG-ODNs reasonable concerns have been raised with regards to their safety, in particular with respect to the potential to trigger autoimmune disorders. Although CpGs are one of the most selective stimulators of dendritic cells with minimal systemic toxicity [65], studies have shown that high doses of bacterial DNA can elicit the production of auto-antibodies against double-stranded DNA in normal mice [66] and accelerate the production of autoimmune antibodies in lupus-prone animals [67]. CpG-ODNs may also facilitate the development of toxic shock by lowering the pathological threshold of lipopolysaccharide (LPS)-tolerance [68]. While

the risks associated with these scenarios are low, they do emphasize the importance of optimization of TLR9 agonists.

2. DESIGN/CREATION OF NOVEL TLR9 AGONISTS

Currently the rational design and optimization of TLR9 agonists is hampered by a lack of basic information on the receptor-ligand interaction as well as the absence of a convenient screening assay. This is largely a consequence of the technical difficulties that are associated with purification of sufficient quantities of the receptor for biochemical characterization. As such, TLR9 ligands are typically evaluated on the basis of their ability to elicit cellular responses in immune cells rather than by their interaction with TLR9. An inherent danger to this approach is that the reduction of numerous potential points of regulation to a single measured output does not permit discrimination of specific events, such as ligand binding, from the remainder of the overall biological process. This is particularly problematic given the emerging evidence that the current paradigm of CpG-mediated activation of the innate immunity likely underestimates the complexity of this system in terms of inputs and functional outputs [15]. Collectively these data highlight the current lack of consensus on TLR9 ligand-binding proclivities as well as the emerging distinction between TLR9 ligand binding and receptor activation.

2.1. TLR9 Sequence Specificity

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

Arguing in favor of sequence-specific binding, an investigation by Rutz *et al.* utilized surface plasmon resonance to investigate ligand binding by a TLR9 fusion protein. Through this investigation it was found that the recombinant TLR9 was able to discriminate not only CpG-containing ODNs, but also higher order motifs that initiate species-specific responses [45], both observations are supportive of the ability of TLR9 to interact with nucleic acids in a highly sequence-specific fashion. In contrast, but equally convincing, is an investigation by Latz in which CpG and 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. In this investigation the authors further demonstrated that GpC-ODNs can inhibit CpG-mediated cellular activation, suggestive that non-stimulatory ODNs compete for the same binding sites on TLR9 [33]. That TLR9 interacts with nucleic acids in a sequence-independent fashion is also supported by an investigation by Yasuda *et al.* where it was shown that endosomally-trafficked phosphodiester ODNs activate TLR9 in a sequence-independent fashion [35]. This was further veri-

fied in this study through surface plasmon resonance investigations that demonstrated that while TLR9 has higher affinity for CpG-ODNs, that GpC-ODNs could become equally effective ligands at higher concentrations [35].

Efforts by our group to elucidate the binding proclivities of TLR9 have reached similar conclusions. Employing a novel agarose shift system that does not require immobilization of the receptor or ligand it was demonstrated that TLR9 has the ability to interact with, and be functionally influenced by, ODNs in a sequence-independent fashion. In contrast, employing more stringent acrylamide electrophoretic shift assays we observed that the ability for TLR9 to discriminate not only the CpG motif, but also higher order sequences of six base pairs in length [69]. The six base pair CpG motif preferential bound by our recombinant human TLR9 protein corresponds with the sequence that elicits the greatest responses in humans *in vivo*. As such the formation of stable complexes is highly sequence-specific; this specificity likely dictates the specificity of activation of the innate immune system.

Similarly, through cell stimulation experiments we observe the exclusive ability for CpG, rather than GpC, PTO-ODNs to activate cellular responses. However this activation can be titrated out with increasing concentrations of GpC PTO-ODNs suggestive that the receptor is able to bind PTO-ODNs in a sequence-independent fashion (Fig. **3**). The stoichiometry of the effect suggests that TLR9 has approximately equal affinity for activating and non-activating sequences. Collectively these results suggest that TLR9 has the ability to interact, and be functionally influenced by nucleic acids in a sequence-independent fashion, while activation is limited to molecules bearing the CpG motif.
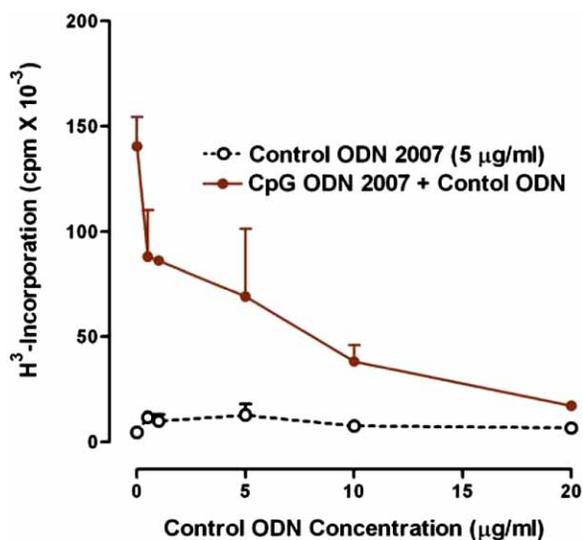
Fig. (3). Cellular Responses of Endogenous TLR9. Purified bovine B cells (2×10^5 cells/well) were co-stimulated with 10 ng/mL recombinant bovine IFN-γ and μg/ml CpG ODN 2007. Increasing concentrations of GpC control ODN were added to the constant concentration of CpG ODN 2007 and cells were incubated for 72 h with H^3-thymidine added during the final 6 h of culture. Data presented are the mean and one standard deviation of values from triplicate cultures.

2.2. Influence of the Phosphothioate Modification

To achieve greater biological stability through nuclease resistance, the majority of TLR9 investigations utilize phosphothioate-(PTO)-modified ODNs. There are however reports to suggest that this is not a neutral substitution, in particular with respect to sequence-specific activation of TLR9 [27, 70]. For example, Rutz demonstrated through surface plasmon resonance that the ability of TLR9 to discriminate higher-order species-specific motifs was limited to natural phosphodiester ODNs as PTO-ODNs were found to be indiscriminate in their ability to bind TLR9, as well as other TLRs [45]. In direct contrast, Roberts [71] found through functional cellular assays that PTO-modified ODNs were associated with higher sequence specificity and that the ability for cells to discriminate the higher-order motifs was dependent upon the PTO-modification. 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 [35, 71]. Our investigation of TLR9-ligand specificity demonstrated that although the ability of the purified receptor to bind plasmid DNA *in vitro* was influenced by both PTO- and PD-ODNs in a sequence-independent fashion, PD-ODNs demonstrated a much stronger cooperative influence. In cell stimulation experiments, however, PTO-ODNs exert a greater cooperative effect, presumably as a result of their increased biological stability [69].

Clearly the PTO-modification is not a neutral substitution, although it is not yet clear how this modification influences 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 [45]. 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 [72-74]. 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 phosphodiester ODN differ from each other" [35].

2.3. Single and Double Stranded Ligands

While it has been demonstrated that innate immune responses can be initiated by plasmid DNA [75, 76] and time course experiments verify that plasmids remain intact within the endosomes for a sufficient duration to serve as TLR9 ligands [77], previous investigations have reached opposing conclusions with respect to the ability of TLR9 to bind double-stranded DNA. While a system established by Cornelie demonstrated effective binding of plasmids [78], Rutz *et al.* reported only a weak interaction between TLR9 and double-stranded ligands [45]. Investigations by our laboratory utilizing the purified ectodomain of human TLR9 may resolve this apparent discrepancy. We have demonstrated that while our purified extracellular domain of TLR9 binds plasmid DNA there is preferential association with the different topological isomers of the plasmid. Specifically, there is preferential binding of forms of the plasmid which are in a super-coiled configuration [69]. Notably in the investigation by Rutz, where they did not observe binding of double-stranded mole-

cules, double-stranded ODNs, rather than plasmids, were employed as ligands. The inability of double-stranded ODNs to form super-coiled species might account for their reduced efficiency as ligands.

2.4. Crystallographic Insights into Ligand Binding

No structural information to offer insights into ligand binding is currently available for the ectodomain of TLR9. However, two independent crystallographic determinations of the ligand-binding domain of TLR3 were simultaneously reported. TLR3 has high sequence conservation to TLR9 and is functionally similar in that it also binds unmethylated, pathogenic nucleic acids within the acidic endosomal compartment. However, rather than resolving the structural mechanisms of nucleic acid recognition, these structures instead added further uncertainty by proposing two conflicting models of ligand binding. Bell *et al.* hypothesized a binding groove for double-stranded RNA within an inner cavity of a TLR3 monomer [79] while Choe *et al.* proposed the convex side of a dimerized structure as the ligand-binding region [80].

2.5. Models of Toll Activation

Investigations of Toll increasingly suggest that activation of this receptor is more complex than initially envisioned. The currently accepted model suggests that Toll is functionally influenced by both ligand-receptor, as well as receptor-receptor, interactions and that activation of Toll involves two non-equivalent binding events that lead to the formation of an active dimer complex [81, 82].

As full length Toll ectodomains are able to form weak dimers in the absence of ligand, the resting state of Toll is suggested to involve ligand-free, inactive dimers. Upon binding of its cytokine ligand Spätzle, these dimers segregate into ligand-free and ligand-bound monomeric subunits. In addition to serving as the ligand Spätzle also functions as a modulatory molecule by exerting an allosteric influence on formation of the active dimer. Specifically Spätzle acts as a homotropic allosteric inhibitor, which upon binding to the active site of the first sub-unit of Toll, induces structural alterations that are translated to the second sub-unit *via* non-covalent associations at the sub-unit interface. The functional consequence of which is to reduce its affinity of the second sub-unit for Spätzle. As a result, the second step of Toll activation occurs with an approximately three-fold lower affinity than the binding of the Spätzle by the first sub-unit, reflective of negative cooperativity [82]. This is proposed as a physiological mechanism to increase the range of responsiveness of Toll (Fig. **4a**). This process also introduces additional potential points of regulation through which various signals could be utilized to influence the equilibrium between the different Toll and Toll-ligand complexes to moderate the sensitivity of the system.

This proposed model of allosteric inhibition in the activation of Toll is supported by the kinetics of cell stimulation. Based on Michaelis-Menton kinetics, in the absence any of allosteric effect, to increase signaling from 10% to 90% requires an approximately 80-fold increase in the concentration of substrate. In the event of positive cooperativity, where the subsequent binding events are favored over the first, this
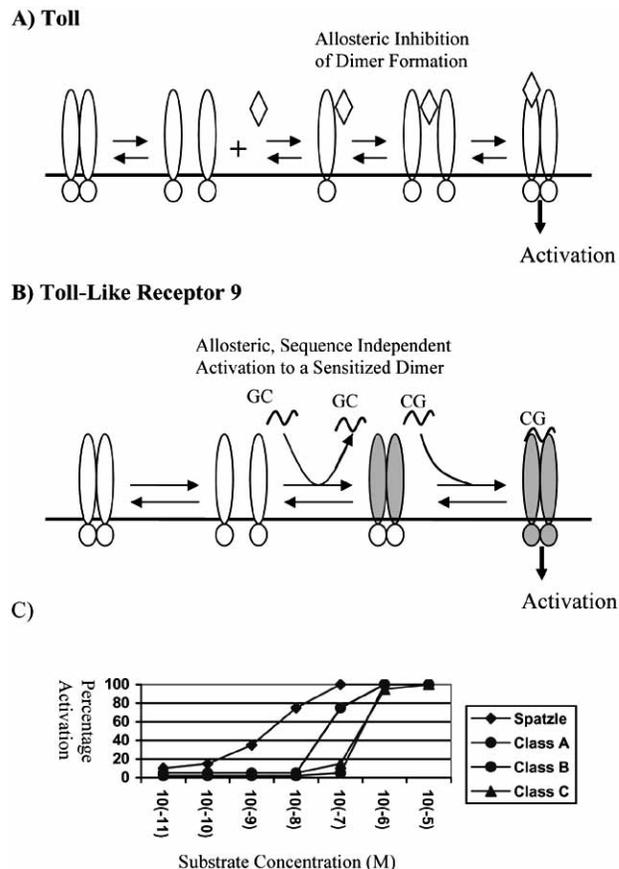
Fig. (4). A) Model of Activation of Toll. Spätlze functions as a homotropic allosteric inhibitor by inducing structural alterations in the second monomer of Toll that discourage ligand binding. **B) Model of Cooperative Activation of TLR9.** Through brief association with TLR9 non-activating nucleic acids are able to function as allosteric activators to promote the formation of ligand-free dimers that have increased avidity for nucleic acids. C) **Kinetics of Activation of Toll and Toll-like Receptor 9:** Activation of Toll occurs over a range of over a four hundred-fold of substrate concentrations, indicative of negative cooperativity. For Toll-like receptor 9, activation by all classes of ODNs occurs in under a ten-fold range of substrate concentrations, indicative of positive cooperativity.

magnitude of activation can be achieved with less than 5-fold increases in substrate concentration. With negative cooperativity, where the second binding event is less favored than the first, up to 400-fold increases in substrate concentration may be required to increase receptor signaling from 10% to 90%. This appears to be the case for Toll, where 300-500 fold increases in Spätzle concentration are necessary to increase signaling activity from 10% to 90% [83] (Fig. **4c**).

2.6. Evidence for Higher-Order Regulation of TLR9

Investigations within our lab also suggest that the equilibrium between TLR9 monomers and dimers plays an important role in regulation of function and that activation of TLR9 is influenced by similar allosteric mechanisms. We have proposed the existence of similar ligand-free dimers as well as differential ligand affinities of different TLR9 com-

plexes. In contrast to the negative cooperativity reported for Toll, we have observed the ability for both single and double-stranded nucleic acids to exert a positive cooperative effect on TLR9 ligand-binding. In the presence of ODNs TLR9 is better able to form nucleoprotein complexes with plasmid molecules and conversely is also better able to bind ODNs when in the presence in plasmid. This mutual cooperativity occurs in a sequence-independent fashion as both CpG and GpC ODNs are equally effective in promoting plasmid binding by TLR9. This is hypothesized to result from the requirement of TLR9 to form dimers to sample potential nucleic acid ligands. This step is likely critical for TLR9 to discriminate activating from non-activating nucleic acids. While non-activating nucleic acids are rapidly released by TLR9 the dimer persists and during this time the ectodomains are sensitized with a higher affinity for nucleic acid binding (Fig. **4b**). The subsequent, and now favored binding, of nucleic acids which contain the activating CpG motif initiate structural alterations that transform the intracellular TIR domains into an active conformation.

The schematic in (Fig. **4b**) illustrates the proposed equilibrium between the various proposed forms of TLR9; monomer, sensitized ligand-free dimer and activated ligand-bound dimer. This model predicts TLR9 to associate with nucleic acids in a sequence-independent fashion, the occurrence of which is becoming increasingly well established. The associations with non-activating ODNs are sufficient to cause dimer formation and shift the monomer-dimer equilibrium of TLR9 to the active form of the receptor.

Others have noted that TLR9 displays "low-affinity" binding to ODNs that is enhanced by the presence of CpG motifs, or increased ODN concentration, to a higher avidity form of the receptor [30]. Furthermore the kinetics of cellular activation by CpG-ODNs support the cooperative model of TLR9 activation both in the sigmoidal nature of the response curve as well as as the magnitude of the substrate concentrations which are required to initiate TLR9 signaling (Fig. **4c**). The cooperative effect of TLR9 activation is observed for all three classes of ODNs suggesting that it functions as a conserved mechanism of activation.

CONCLUSIONS

Collectively these results demonstrate the structural and functional complexity of the various TLR9 agonists as well as the current uncertainty of many the binding tendencies of this receptor. From this uncertainty a model is emerging that incorporates the ability for TLR9 to bind and be influenced by nucleic acids in both sequence-dependent and sequence-independent fashion. While ligand-binding for activation of the system is highly sequence-specific, non-activating nucleic acids are able to modulate TLR9 responsiveness through sensitization of the receptor. The physiological significance of this allosteric effect may be in determining the set point for TLR9 signaling. That increased concentrations of nucleic acid are able to sensitize the system in a sequence-independent fashion might be of physiological significance following the phagosomal ingestion of bacteria with the subsequent release of microbial DNA "priming" the TLR9 system for subsequent activation by nucleic acids bearing the CpG motif. This dual requirement for maximal activation

may function as a safe guard to prevent the induction of inappropriate innate immune responses.

The observation that TLR9 can be functionally influenced by non-CpG elements expands the range of nucleic acids that can be employed to modulate innate immune responses through TLR9. The design of novel TLR9 immunotherapeutics may benefit from consideration of TLR9 modulators, rather than direct activators. These molecules would be selected on the basis of their ability to "prime" the TLR9 system for CpG-specific activation with the potential for co-formulation of these activating and modulating molecules. The structural requirements for these modulating molecules are still unclear. Comparable to much of the uncertainty that exists for the TLR9 agonists it is likely that unique sequence and structural features, as well as pH-dependent changes in protonation state likely influence the specificity of binding as well as the resulting functional consequences.

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