

Modulating Toll-like Receptor 7 and 9 Responses as Therapy for Allergy and Autoimmunity

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Abstract Type I allergic diseases, such as allergic rhinitis and asthma, depend on allergen-induced T-helper type 2 (Th2) cells and IgE-secreting plasma cells. Fortunately, this harmful immune response can be modified by engaging Toll-like receptor (TLR)7 and TLR9, offering hopes to allergy sufferers. While clinical trials employing synthetic ligands for TLR7 or TLR9 are under way, one can wonder whether TLR7 or TLR9 engagements may trigger inadvertent autoreactivity and/or Th1-/Th17-mediated tissue pathology. To neutralize such danger, we have pioneered the development of potent TLR9 pathway antagonists, inhibitory oligonucleotides (INH-ODNs), which work in a sequence-specific manner. Interestingly, INH-ODNs also have TLR7-inhibitory properties; however, these effects appear to be sequence independent and phosphorothioate backbone dependent. In B cells, co-engagement of the B-cell receptor for antigen and TLR7 or TLR9 may influence how INH-ODNs impose their regulatory effects. INH-ODNs block TLR9 activation by competitively antagonizing

ligand binding to proteolytically cleaved C-terminal TLR9 fragment. One may envision future use of INH-ODNs in systemic autoimmune diseases, DNA-mediated sepsis, or other situations in which chronic inflammation results from abnormal TLR7- and/or TLR9-mediated immune activation.

Keywords Toll-like receptor · TLR7 · TLR9 · CpG-ODN · Bacterial DNA · INH-ODN · R-848 · Systemic lupus erythematosus · SLE · Asthma · Allergy · Autoimmunity · Ragweed · Endosomes · Th1 · Th2 · Th17 · Regulatory T cells · B cells · IgE · Cathepsins · Endopeptidases · Autophagosomes · Animal models of lupus

Introduction

Extrinsic asthma, allergic rhinitis, drug-induced anaphylaxis, and other allergic diseases are dominated by proinflammatory T-helper type 2 (Th2)- and IgE-mediated responses against innocuous antigens in atopic individuals [1]. As Th2 cells can induce, regulatory T cells can suppress several features of allergic inflammation [2]. Reduced risk of atopic disease is associated with early-life exposure to infections and microbes, a concept well-known as the *hygiene hypothesis*. This benefit from microbial exposure may reflect alterations in host innate immune reactivity. One explanation is that signals through the pathogen-associated molecular patterns (eg, through Toll-like receptor [TLR]7 or TLR9, or via NOD-like proteins) may confer protection against allergic disorders by priming antigen-presenting cells to produce cytokines favoring a Th1-type immune responses, and thus preventing the development of deleterious Th2 response [3, 4]. However, on the contrary, persistent activation of our

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innate immune system through nucleic acid-sensing TLRs may, unfortunately, cause harm, resulting in systemic autoimmunity. Therefore, one needs to develop a strategy for how to counteract undesired TLR7 and TLR9 signaling to prevent/reverse possible pathology.

Therapeutic Benefits and Possible Side Effects of Using Toll-like Receptor 7 and Toll-like Receptor 9 Agonists for the Treatment of Allergic Type Hypersensitivity

In accordance with the hygiene theory, exposure of suckling mice to influenza infection provided a TLR7- and natural killer T (NKT)-/forkhead box P3⁺ regulatory T-cell-dependent protection against asthma during their later adult life [5•]. Further supporting this concept, researchers have shown that NKT cells expressed TLR7 and that the protection against asthma depended on their ability to make interferon (IFN)- γ [6]. Thus, manipulating the immune system through induction of regulatory T cells, switch to Th1 response, or direct tolerance-induction in allergen-reactive T cells may potentially reprogram the aberrant immune system in favor of allergy. Oral hyposensitization to allergens probably works through the induction of allergen-specific regulatory T cells but is unpredictable and is beneficial in only a portion of patients [7]. On the other hand, TLR7 and TLR9 ligands can directly and more predictably induce B cells to switch to IgA and block CD40⁺ interleukin (IL)-4 (or lipopolysaccharide-positive IL-4)-induced IgE secretion [8]. Interestingly, TLR7 function is reduced in adolescents with asthma, which may contribute to their susceptibility to respiratory viral infections [9].

TLR9 ligands (ie, oligonucleotides containing unmethylated CpG sequences [CpG-ODNs]) were able to prevent and reverse antigen-induced eosinophilic airway inflammation in animal models of asthma [10]. Preclinical models of asthma have demonstrated that CpG-ODNs can also reduce circulating levels of IgE together with bronchial hyperactivity. In models of chronic allergen exposure, CpG-ODNs were also effective at preventing development of airway remodeling [11], while in established asthma, TLR9 ligands could reverse manifestations of asthma when used alone or in combination with allergen hyposensitization [12, 13]. TLR9 activation induces a very strong Th1-like pattern of cytokine release. However, Th1 cytokines do not appear to be necessary for the therapeutic benefit in preclinical models of atopic asthma [14]. Additional mechanisms may involve induction of regulatory-type responses (involving IL-10 release) [15] and expression of tryptophan catabolic enzyme indoleamine 2, 3-dioxygenase [16].

In addition to TLR9 ligands, TLR3 and TLR7/8 ligands are also promising candidate drugs for treatment of allergic asthma and rhinitis. Systemic application of synthetic ligands for TLR3 or TLR7, such as polycytidylic-polyinosinic acid (p[I:C]) and R-848, respectively, during the sensitization phase prevented the production of allergen (ovalbumin)-specific IgE and IgG₁ antibodies and subsequently abolished all features of experimental asthma, including airway hyperresponsiveness and allergic airway inflammation, in animal models of asthma [17]. Furthermore, administration of p(I:C) or R-848 in animals with already established primary allergic responses caused a reduction in the secondary response following aerosol rechallenges. This effect appeared to be dependent on both IL-12p35 and IL-10 [17]. The way in which TLR7 ligands manipulate allergy may further depend on their ability to block IL-17 and IL-13 production via an early B-cell-derived regulatory cytokine IL-10 and via a later effector T-cell-derived IL-10 [18•]. Controversially, TLR7 stimulation of IFN- α -producing plasmacytoid dendritic cells could by itself induce a strong Th17 response [19•], therefore challenging the above concept. Treatment of asthmatic transgenic mice with the TLR7 ligand resiquimod increased the expression of secretory leukocyte protease inhibitor (SLPI) and simultaneously decreased the lung inflammation. Interestingly, although SLPI expression correlated with the lung protection, treatment with the TLR7 ligand was still effective in asthmatic SLPI knockout mice. Thus, although the expression of SLPI, as seen in transgenic asthma models, provides protection against allergic inflammation, treatment with resiquimod appears to work independently of the SLPI expression [20].

Human clinical trials using TLR9 ligands are ongoing [1, 3, 4]. Early clinical trials have yielded mixed results, including a significant benefit when CpG-ODNs were conjugated to ragweed allergen in an allergic rhinitis protocol, but only limited efficacy in asthmatics when a TLR9 ligand was administered prior to allergen challenge [21]. Clinical application of TLR7 and TLR9 ligands depends on developing new strategies to improve the delivery of these drugs into the lung. For example, intranasal administration of the TLR7/8 ligand R-848 in experimental animals induced a transient immune response characterized by type 1 IFN production and infiltration of innate immune cells into the lung. This conferred long-term suppression of allergic airway disease via two complementary mechanisms, one mediated by type 1 IFNs that provided acute protection by directly inhibiting effector Th2 responses, and one mediated by immunoregulatory CD8⁺ T cells, causing a long-lasting protection by suppressing Th2 responses in an IFN- γ -dependent manner [22]. One important difference between the TLR7 and TLR9 agonists is that TLR7 agonists

appear to have a stronger antiasthmatic potential with the lowest proinflammatory profile when compared with locally administered TLR9 ligands [23].

In addition to their use in allergy, TLR7/8 and TLR9 ligands have been studied in animal models of infection and cancer (Fig. 1). As vaccine adjuvants, these agents provided the best co-stimulus when directly conjugated to the antigen [24•] Systemic administration of TLR7 agonists is effective in suppressing Th2-derived inflammation; however, with systemic induction of various cytokines (eg, IL-6, IL-12, and type 1 IFN), lymphadenopathy and liver damage are also observed [25, 26]. Furthermore, in animal models of lupus, administration of potent TLR3, TLR7, or TLR9 ligands could worsen spontaneous autoimmunity and shorten survival of experimental animals [27–30], suggesting that the abnormal induction or persistence of TLR3, TLR7, or TLR9 activation may be as harmful as the allergy itself. Duplication of TLR7, as seen in male BXSB mice, which develop a spontaneous lupus-like disease, is solely responsible for the phenotype [31]. Therefore, we need a strategy to manipulate and counteract these potentially harmful TLR7- and TLR9-mediated responses. Fortunately, as discussed below, antagonists for TLR7 and TLR9 activation have been developed recently and are currently undergoing phase 1 clinical trials.

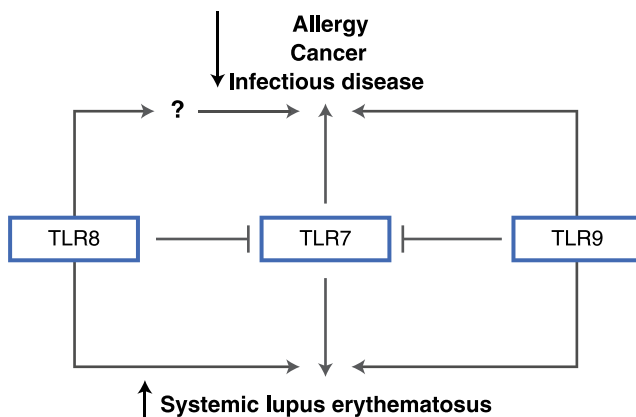


Fig. 1 Toll-like receptor (TLR)7: a key molecule at the crossroads between allergy and autoimmunity. Activation through nucleic acid-sensing receptors TLR7 and TLR9 may benefit patients with established allergy. Synthetic TLR7 or TLR9 ligands have strong vaccine adjuvant properties and may induce potent T-helper type 1-directed T-cell responses. Similar mechanisms may be operative in TLR7- and/or TLR9-mediated cancer protection. In contrast, uncontrolled TLR7 activation may result in systemic autoimmunity as observed in BXSB male mice that have duplication in the *TLR7* gene and develop spontaneous lupus. TLR8 controls TLR7 stimulation, and lack of TLR8 may result in TLR7-mediated pathology and spontaneous autoimmunity. TLR9 may have both regulatory (by controlling TLR7 activation) and proinflammatory properties; however, in healthy mice, lack of TLR8 does not promote significant autoimmunity

Toll-like Receptors and Systemic Autoimmunity

A detailed review of the role of TLR7 and TLR9 in the induction of autoimmunity is beyond the scope of this article, and readers are referred to several excellent reviews covering this topic in more detail [32–35, 36•, 37–39]. Complexes of nucleic acids and associated proteins are frequently targeted by autoimmune B cells in patients with systemic autoimmune diseases [32, 36•]. For example, patients with systemic lupus erythematosus (SLE) develop autoantibodies against nucleosomes/chromatin, DNA-binding protein Ku, ribosomal P proteins, and small nuclear ribonucleoproteins (eg, Sm and RNP). Those with Sjögren's syndrome have autoantibodies against components of the spliceosomal machinery (SS-A/Ro and SS-B/La), while the non-Sm component of the small nuclear ribonucleoprotein is targeted in mixed connective tissue disease. Centromeres and nucleolar antigens are recognized by autoantibodies in limited and diffuse systemic sclerosis, respectively. Finally, histidyl tRNA synthetase (Jo-1) and similar enzymes are targeted in polymyositis and in patients with so-called antisynthetase syndromes [40]. What is common to all these autoantigens is that the target autoantigen frequently makes a part of a larger macromolecular complex associated with RNA or DNA. Over the past 10 years, it has become clear that the immunogenicity of these macromolecules depends on cognate recognition of associated nucleic acids by TLR7, which recognizes single-stranded RNA [41], or TLR9, which recognizes hypomethylated CpG-DNA, together with the B-cell receptor-mediated recognition of the protein component [32, 36•, 42, 43]. TLR8, which also recognizes single-stranded RNA, may play a regulatory role in autoimmunity, as its deficiency clearly leads to autoimmunity in mice [44••]. It appears that TLR8 may keep TLR7-induced immune activation in place, as TLR8^{-/-} mice are hyperresponsive to various TLR7 ligands and make autoantibodies against double-stranded DNA and small ribonucleoproteins. They also develop clinically significant glomerulonephritis. Interestingly, this phenotype is lost in double-knockout TLR7^{-/-}TLR8^{-/-} mice. A similar regulatory role has been suggested for TLR9 [45–47]; however, mice lacking TLR9 do not develop spontaneous autoimmune disease [48], but TLR9^{-/-} strains of mice predisposed to develop lupus (eg, MRL-Fas^{lpr/lpr}) have a much shorter life span and suffer from a worsening kidney disease, fueling controversy about the role of TLR9 in autoimmunity [46, 47]. Again, this phenotype is highly influenced by abnormal signaling through the TLR7 [49, 50•]. Interestingly, although antibodies against double-stranded DNA correlate nicely with lupus flares and particularly with the worsening of lupus nephritis, the lack of TLR9 ameliorates anti-double-stranded DNA antibody production in lupus mice [47].

This may suggest that these antibodies are just an epiphenomenon, or that IgM anti-double-stranded DNA antibodies may have an important, previously unrecognized protective role in clearing circulating DNA.

It is now well-established that activation of self-reactive B cells expressing low-affinity receptors for DNA or RNA containing autoantigens depends on a secondary signal delivered through TLR9 or TLR7, respectively. This was first shown by Lau et al. [41] and later confirmed by others [42, 51, 52]. They used AM14 B cells derived from lupus mice that express a low-affinity rheumatoid factor-like B-cell receptor, which preferentially recognizes IgG_{2a} antibodies complexed with the self-antigen. However, cell proliferation only occurs when CpG-DNA is part of this complex and when it is delivered to an endosomal compartment via a B-cell receptor-dependent route, providing a co-stimulatory signal to the cell. Similarly, RNA-containing immune complexes induce AM14 B cells to proliferate, apparently in a TLR7-dependent manner [41].

Apoptotic cells are the most important (but not exclusive) resource of autoantigens for systemic autoimmunity [53]. They express autoantigens such as nucleosomes on their surfaces in the form of apoptotic blebs [54, 55]. However, this is not restricted to nucleosomes, as other nucleic acid-containing autoantigens may gain access to the cell surface at various times during programmed cell death. Eventually they become released into the surrounding milieu and are subsequently cleared by macrophages and other phagocytic cells. This finely orchestrated mechanism is obviously disarranged in lupus due to enhanced production of autoantigens or their less efficient disposal, resulting in their longer persistence in circulation and higher probability to engage low-affinity autoreactive B cells [56–58].

Therefore, TLRs, particularly TLR7 and possibly TLR9, are legible targets for pharmacologic inhibition in the treatment of systemic autoimmunity in humans. Indeed, inhibition of these two pathways, particularly TLR7, benefited animal lupus [38, 59•, 60–64, 65•].

Short Oligonucleotides as Regulators of Toll-like Receptor 9- and Toll-like Receptor 7-Induced Activation

In their attempt to understand what makes bacterial DNA [66, 67], but not our own DNA, immunostimulatory, Halpern and Pisetsky [68] discovered that synthetic ODNs containing long stretches of guanosine nucleotides could block bacterial DNA-induced immune activation when used over the micromolar concentration range. However, their inhibitory activity was not limited to bacterial DNA-induced activation, as they also could block T-cell

activation induced by lectins and other powerful mitogens [69, 70]. It was later discovered that inhibitory DNA sequences could be found in certain adenoviral strains [71]. When TLR9 was discovered as a receptor for hypomethylated CpG-DNA [48], several groups focused their attention on studying the interaction between the ligand and its receptor. It was found that TLR9 originally resides in the endoplasmic reticulum (ER) and that upon ligand entry, it translocates to an endolysosomal compartment, where it undergoes final proteolytic cleavage, generating an active receptor [72–79, 80•, 81•].

Our major objective was to determine the optimal structural requirements for inhibition of bacterial DNA- and unmethylated CpG-ODN-induced TLR9 activation in rodents [82–84] and humans [85, 86•]. We also wanted to correlate their biologic activity with the affinity for the TLR9 [86•]. Based on these studies, inhibitory oligonucleotides (INH-ODNs) were discovered with approximately 1,000-fold higher potency compared with the original poly G sequences [84, 87–91]. Interestingly, besides a need for a stretch of three to five consecutive G nucleotides, there was an additional requirement for a pyrimidine-rich doublet/triplet, optimally CCT, placed three to six nucleotides upstream of the G-rich block [59•, 82, 92•, 93]. Lack of either of these two elements made INH-ODNs at least 100-fold less potent. Moreover, the nucleotide sequence linking these two blocks could finely tune INH-ODN activity, but no more than twofold to fivefold [83]. Both inhibitory blocks had to be simultaneously present on the same molecule, and their orientation had to be in a proper order (ie, GGG → CCT) [82]. These structural requirements for optimal TLR9 inhibition were found in ODNs carrying the natural phosphodiester (PO) backbone, as well as in those made with the phosphorothioate (PS) backbone [86•, 88]. The later backbone is used in clinical trials to provide resistance against serum nucleases, thus allowing ODNs a longer half-life in vivo. The caveat is that (PO)ODNs require at least 100-fold higher concentration to mediate equivalent inhibitory potency compared with (PS)ODNs, and this was true only when (PO)CpG-ODNs were used for stimulation. On the contrary, (PO)INH-ODNs are incapable of blocking powerful (PS)CpG-ODN-induced activation [86•]. A possible explanation is that the PS backbone binds TLR9 with much higher affinity compared with the natural PO backbone, a hypothesis recently raised by Haas et al. [94]. These structural requirements were found to be optimal for TLR9-expressing murine B cells, macrophages, and dendritic cells, as well as for human peripheral blood mononuclear cells, purified B cells, and B-cell lines [59•, 82, 83, 86•]. Furthermore, these requirements were validated by several other groups [88, 90, 91, 95]. Interestingly, telomeric ends of mammalian chromosomes contain repetitive stretches of TTAGGG hexamers. Synthetic ODNs containing these

motifs were developed by Gursel et al. [96] and were found to have effects in animal models of lupus, pneumonitis, and sepsis [61, 97, 98]. It was also shown that these ODNs could prevent cancer formation in a model of chronic inflammation-induced carcinogenesis [99].

In a human system, like in rodents, the same requirements for CC(T) triplets and three to five consecutive Gs properly spaced and oriented are necessary for optimal TLR9 inhibition, as seen in primary B cells and B-cell lines [86•]. The shortest INH-ODNs with the inhibitory potency in the nanomolar range were 10 nucleotides long. However, extending INH-ODNs for a few nucleotides at their 5′ end further increased their inhibitory potency for human B cells in a manner that appeared to be largely sequence independent but length dependent [86•].

As TLR9 undergoes spontaneous dimerization even in the absence of an external ligand [100], we studied how ODN aggregation and 5′ loop formation affects INH-ODNs' ability to block TLR9 activation [101]. We further questioned whether INH-ODNs can complex with stimulatory ODNs similarly to what was recently shown for chloroquine, which blocks TLR9-induced activation at least in part by directly binding nucleic acids [102•], and not through the widely believed interference with the endosomal acidification [103]. Similar to others, we found that the ability of INH-ODNs to make G4 stacks was not necessary for their TLR9 inhibitory potency [84, 90, 91, 101]. There was no relationship between the INH-ODN activity and self-association at endosomal pH and no evidence of direct binding between INH-ODNs and stimulatory CpG-ODNs. Finally, the 5′ loop formation was also not required, as INH-ODNs with the preserved stem loop formation but with a disrupted stretch of three consecutive G nucleotides lost their inhibitory activity [101]. Therefore, we concluded that certain features of the primary linear sequence are critical for TLR9 inhibition.

A more complex inhibitory pattern was observed when INH-ODNs were synthesized with complete palindromic sequences, or with short 5′ or 3′ overhangs (class R INH-ODNs) [59•, 92•]. Depending on experimental conditions, INH-ODNs with full palindromic sequences could make hairpins or, in the presence of ions, dimers [92•]. Whereas in biologic assays, hairpins and dimers had similar inhibitory potency for TLR9-induced activation, we surprisingly discovered profound cell-type-dependent differences in their inhibitory activity when compared with linear INH-ODNs [59•]. To minimize INH-ODNs' ability to make stacks, we only used INH-ODNs with short 3 G stretches. Whereas in macrophages/dendritic cells, linear and palindromic ODNs had very similar potency for complex CpG-ODN-induced TLR9 activation, in primary human and murine B cells and B-cell lines stimulated with linear CpG-ODNs, linear

inhibitory ODNs were between 10- and 100-fold more potent. Interestingly, when the B-cell receptor for antigen was simultaneously co-engaged with the TLR9, the potency of palindromic INH-ODNs increased at least 10-fold and equalized that of linear INH-ODNs [59•, 104]. This observation was true in model systems in which INH-ODNs were delivered to an intracellular endolysosomal compartment via a presumed B-cell receptor-mediated entry [59•] or, alternatively, through a non-B-cell receptor-mediated uptake [104]. The increased INH-ODN potency upon B-cell receptor engagement was outcome dependent and was observed for B-cell proliferation in autoreactive rheumatoid factor-specific B cells, as well as for G1-M entry and apoptosis protection in polyclonal murine B cells. However, the difference in IL-6 secretion remained 10-fold in favor of linear INH-ODNs, even after co-engaging the B-cell receptor, suggesting that the B-cell receptor signal may differentially affect the intracellular trafficking of INH-ODNs [104]. Interestingly, when B-cell receptor and TLR9 interaction was studied in stimulatory models, in addition to their synergistic effect on cellular activation, certain new outcomes (eg, B-cell-derived IL-2) and RANTES (regulated on activation, normal T-cell expressed and secreted) expression were observed only when both receptors were simultaneously co-engaged [105–107]. Thus, the B-cell receptor signal may modify TLR9-induced B-cell activation (and inhibition), which may, at least in part, be due to enhanced autophagosome formation [108], allowing more efficient signal transduction. On the other hand, the 10-fold potency difference seen with class R palindromic INH-ODNs for TLR9-engaged versus TLR9-plus-B-cell receptor-engaged B cells may be of crucial importance for targeted inhibition of self-antigen (DNA) activated B cells, a strategy that may be foreseen as a therapy for SLE.

Direct Binding to Full-Length Toll-like Receptor 9 Does Not Correlate with Oligonucleotides' Inhibitory Potency

Nucleic acid-sensing receptors of the TLR family (eg, TLR3, TLR7, and TLR9) are very tightly regulated to prevent bystander activation by our own DNA [37]. First level of regulation is related to their intracellular localization. As directly shown for TLR9, and by analogy suggested for TLR3 and TLR7, in dendritic cells and probably in B cells, full-length TLR9 is initially sequestered within the ER [74]. When CpG-DNA enters a cell, it sends a signal to TLR9 to translocate to an endolysosomal compartment, where the subsequent interaction takes place. TLR9 most likely fails to reach the cell membrane, which is in contrast to other TLRs, such as TLR2 and TLR4. TLR9 translocation from the ER requires help from the UNC93b1 adapter molecule, as cells lacking this key molecule have

deficient TLR3-, TLR7-, and TLR9-mediated signaling [75–77]. Once TLR9 reaches the endosomal compartment, it undergoes enzymatic cleavage into a C-terminal TLR9 fragment. Although it is still a mystery which enzyme catalyzes TLR9 cleavage (caspases, endopeptidases, or both) [78, 79, 80•, 81•], it is well-established that the cleaved product, not the full length TLR9, preferentially binds CpG-DNA and is functionally active [79]. Binding of CpG-DNA to dimeric TLR9 leads to its conformational change [100], resulting in activation of MyD88-dependent signaling pathways, ultimately causing mitogen-activated protein kinase/AP1 and nuclear factor- κ B nuclear translocation and subsequent gene transcription [109, 110].

It appears that the full-length TLR9 indistinguishably binds PS-modified synthetic ODNs, including INH-ODNs [74, 111]. By contrast, when natural CpG-ODNs with the PO backbone were compared with functionally inactive GpC-ODNs, they bound TLR9 with much higher avidity [111]. However, the primary force leading to productive DNA/TLR9 interaction appears to be the sugar (deoxyribose) backbone of DNA [94]. To clarify whether binding affinity of (PO)–INH-ODNs translates into their biologic activity, we, in collaboration with Latz, used an α -screen binding assay to measure the (PO)–INH-ODNs interaction with the full-length TLR9. We carefully chose INH-ODN variants with a 500 potency range in functional assays. Tested ODNs had a very similar cellular uptake, ruling out nonspecific competition for the cellular entry. Surprisingly, only a 2.5-fold range in binding affinities was observed, suggesting an alternative explanation for biologic differences [86•].

Inhibitory Oligonucleotides, But Not Control Oligonucleotides, Bind to Cleaved C-terminal Toll-like Receptor 9 with Higher Affinity and Compete with Oligonucleotides Containing Unmethylated CpG Sequences

Avalos and Ploegh [112•] recently published a paper that suggested a solution to the puzzle regarding the interrelationship between ODN structure, TLR9 binding, and their functional activity. Using murine RAW 264.7 cells stably transfected with the TLR9-Myc and also confirmed in bone marrow–derived dendritic cells from TLR9^{−/−} mice, they concluded the following:

1. Cleaved, but not full-length TLR9, preferentially binds CpG and INH-ODNs. C-terminal TLR9 binds control GpC-ODNs, but with much lower affinity. This applies to ODNs with a PO or PS backbone.
2. At least two different INH-ODNs, including a prototypic INH-ODN 2088, but not the control ODN, were capable of displacing CpG-ODNs from their binding to

the C-terminal TLR9, as shown in ex vivo competition assays. This competition was independent of the ODNs' ability to compete for the cellular uptake.

3. Pulse-chase experiments have shown that neither CpG-ODNs nor INH-ODNs can affect TLR9 cleavage.

The authors therefore concluded that the ligand binding to the C-terminal TLR9 fragment determines the outcome of activation through the TLR9. How it actually works—through the disaggregation of TLR9, prevention of the conformational change in TLR9, or by masking the critical docking site on TLR9—appears likely to be determined in future studies. Unfortunately, these studies have not utilized INH-ODN variants in which the canonical building blocks (CCT and GGG) were deleted or synthesized in a reverse order. The later ODNs with the reverse order would be expected to have similar binding affinity for TLR9. However, it is noteworthy that C-terminal TLR9 appears to be able to discriminate between the orientation of the CpG motif and the inhibitory motif, and, more importantly, sole binding to TLR9 is not sufficient for cellular activation. Another caveat from these studies was that they could not distinguish the contribution from the G quartets, as both INH-ODNs tested, unfortunately, had the ability to make significant G4 stacks, which could have interfered with the CpG-ODN binding simply due to steric hindrance. Another unfortunate setback from the current study was that the authors failed to test whether INH-ODNs with the natural PO backbone could displace biotin-labeled stimulatory (PO)CpG-ODNs from their binding to cleaved TLR9. Finally, although the TLR9 C-fragment remains the prime candidate for TLR9-mediated downstream activation, others have shown that mutations in the N-terminal domain of TLR9 may render TLR9 inactive [113]. Whether this later effect involves a control of TLR9 folding or some other mechanism remains to be determined. To resolve this issue, we must await results from crystallographic studies utilizing TLR9 in the absence or presence of its natural (PO) or synthetic (PS) agonists and antagonists.

TGC Motifs as Toll-like Receptor 7 Inhibitors

Barrat et al. [107] first noticed that adding a single or multiple GC flips to an ODN could convert them into potent TLR7 inhibitors. IRS-661 is such an INH-ODN with a palindromic core containing five consecutive GC flips. It behaved as a strong TLR7 inhibitor in both B cells and dendritic cells. Furthermore, it was able to block spontaneous animal lupus in the MRI-Fas^{lpr/lpr} strain of mice [63]. When a single TGC motif was added to the 5' end

of a prototypic TLR9 inhibitor, it converted this INH-ODN into a dual TLR7/9 inhibitor capable of blocking TLR7- or TLR9-mediated activation both in vitro and in vivo [107]. Their prototypic TLR7/9 inhibitor also worked well in the NZB/W-F1 mice that develop a spontaneous lupus-like disease [62]. However, it is not clear whether the observed effect in experimental animals was mediated through TLR7, TLR9, or both. The whole concept that a single TGC motif can convert an ODN into a potent TLR7 inhibitor has been challenged by several groups, including our own, and awaits further clarification [59, 114, 115]. For example, studies in primary B cells have failed to show any enhancement of TLR7 inhibitory activity when 32-mer INH-ODNs were synthesized with a 5' TGC motif. In our hands, any 32-mer ODN made with a PS backbone could inhibit TLR7 activation with potency in a middle/upper nanomolar range [116]. However, contrasting a single TGC motif, INH-ODNs containing multiple TGC motifs wrapped in a palindromic core were more potent in B cells. Interestingly, when the B-cell receptor and TLR7 were co-engaged in B cells, we observed a significant loss of inhibitory potency of synthetic ODNs for TLR7-mediated activation, an obstacle that must be accounted for in future human clinical trials [116].

First Human Trial of Inhibitory Nucleotides in Lupus

Reflecting the progress made in preclinical studies, Dynavax Technologies Corp. (Berkeley, CA), with its strategic partner GlaxoSmithKline (GSK, Research Triangle Park, NC), announced on April 20, 2011, that it will start a phase 1 human clinical trial in lupus. DV1179, an inhibitor of TLR7 and TLR9, will be assessed in multiple ascending doses. A total of 24 healthy individuals will each receive 4 weekly injections of DV1179. Following successful completion of the trial, Dynavax expects to initiate a proof-of-concept study in lupus patients.

Conclusions

TLR7 or TLR9 agonists are currently tested for efficacy and safety in clinical trials for allergic rhinitis and asthma. On the contrary, TLR7 and/or TLR9 antagonists, similar to antimalarics (eg, chloroquine), may provide benefits to patients with SLE or other systemic diseases in which persistent co-activation through TLR7 and/or TLR9 contributes to pathology.

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- Of major importance

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