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Commentary

# Cooperative activation of human TLR9 and consequences for the clinical development of antisense and CpG oligodeoxynucleotides

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In their current work, Pollak and colleagues[1] made an important contribution with regard to immunostimulatory properties of antisense oligodeoxynucleotides (ODNs), which as a general rule need to be excluded when specific antisense-mediated silencing of the target gene is intended. The notion that antisense ODNs can exert immunostimulatory effects has co-evolved with the field of antisense from its early days. The observation of immunostimulatory activity of certain sequences was made soon after phosphorothioate oligonucleotides were introduced to achieve antisense-mediated gene silencing.[2–4] In fact, in this context, TLR9 was the first Toll-like receptor (TLR) reported to be involved in immune sensing of a nucleic acid.[5] TLR9 activation in B cells by oligonucleotides containing unmethylated CpG motifs in species-specific sequence contexts (CpG ODNs) and the resulting clinical potential of CpG ODNs as humoral immune adjuvant was recognized early on.[6] Insight in the contributions of sequence, structure formation, and backbone modification on the functional outcome in the primary immune cell types involved (human B cells and plasmacytoid dendritic cells) led to the establishment of different classes of CpG oligonucleotides (CpG-A, CpG-B, CpG-C), which have been routinely used for TLR9 activation until today.[7] However, the molecular mechanism of binding of CpG ODNs to TLR9 remained elusive until a series of brilliant structural studies revealed two sequence-dependent specific single-stranded DNA (ssDNA) binding sites (CpG and xCx) to be involved in the dimerization and activation of TLR9 and, furthermore, the existence of another binding site for ssDNA that mediates inhibition of TLR9 activation by inhibitory oligonucleotides.[8,9] In this context, it is important to keep in mind that in those studies, (1) most structural information is derived from TLR9 of species other than human, (2) structural studies were performed with unmodified phosphodiester oligonucleotides, while phosphorothioate oligonucleotides were used in cell culture experiments because of their higher nuclease stability, and thus, (3) the role of DNase degradation providing short single-stranded DNA ligand structures from single- and double-stranded DNA as it may occur in biological systems is not addressed. The involvement of nucleases in the provision of specific ligands for TLR9 (DNase II) and for TLR7 and TLR8 (RNase T2 and RNase 2) in the acidic environment of the endolysosome of immune cells adds an additional layer of complexity to the physiological roles of TLR7, TLR8, and TLR9 where the functional forms (after pH-dependent proteolytic processing) of TLR7, TLR8, and TLR9 are localized.

The current view includes the following four binding modes of ssDNA to TLR9[8,9]: binding of a ssDNA to the CpG binding site but not the xCx binding site (weak activation) (mode 1); binding one type of ssDNA to the CpG binding site and a second type of ssDNA to the xCx binding site (augmented activation) (mode 2, corresponding to model 2 of cooperative binding in Pollak et al.[1]); binding of one type of ssDNA with one motif binding to the CpG site and another molecule of the same type of ssDNA binding to the xCx binding site with a second sequence motif (augmented activation) (mode 3, corresponding to model 1 of cooperative binding in Pollak et al.); and one molecule of one type of ssDNA simultaneously binding to both the CpG site and the xCx binding site of TLR9 (augmented activation) (mode 4) (see Figure 1). All four binding modes may exist and occur under certain circumstances.

The improved understanding of TLR9-mediated recognition of DNA over the years guided the design of antisense oligonucleotides that are capable to specifically silence their target gene without stimulating immune responses via TLR9, although under certain circumstances, a combination of antisense and immunostimulation might be desirable.[10] Many antisense oligonucleotides are currently in clinical development, and a number of them have already been approved as drugs, highlighting the great success of antisense oligonucleotides as a novel therapeutic entity.[11] Nevertheless, some of the antisense oligonucleotides being developed still show unexpected immunostimulatory properties. In this context, the work by Pollak and colleagues represents a great step forward in the mechanistic understanding of such unexpected immune activities. The work not only provides important new molecular insight, but it also offers solutions on how such unexpected properties of antisense oligonucleotides can be analyzed and avoided early on.

Of the four binding modes 1 to 4 above, Pollak and colleagues demonstrate the existence of a cooperative type of TLR9 activation by two non-CpG-containing antisense oligonucleotides resembling mode 2 (model 2 of cooperative binding in Pollak et al.[1]). In this cooperative mode, two different (non-CpG containing) ssDNA molecules, such as the ODN 95 combined with ODN 18 or ODN 05 (Pollak et al., Figure 2), bind to TLR9 in a non-competitive fashion to different sites (Pollak et al., Figure 3). Only

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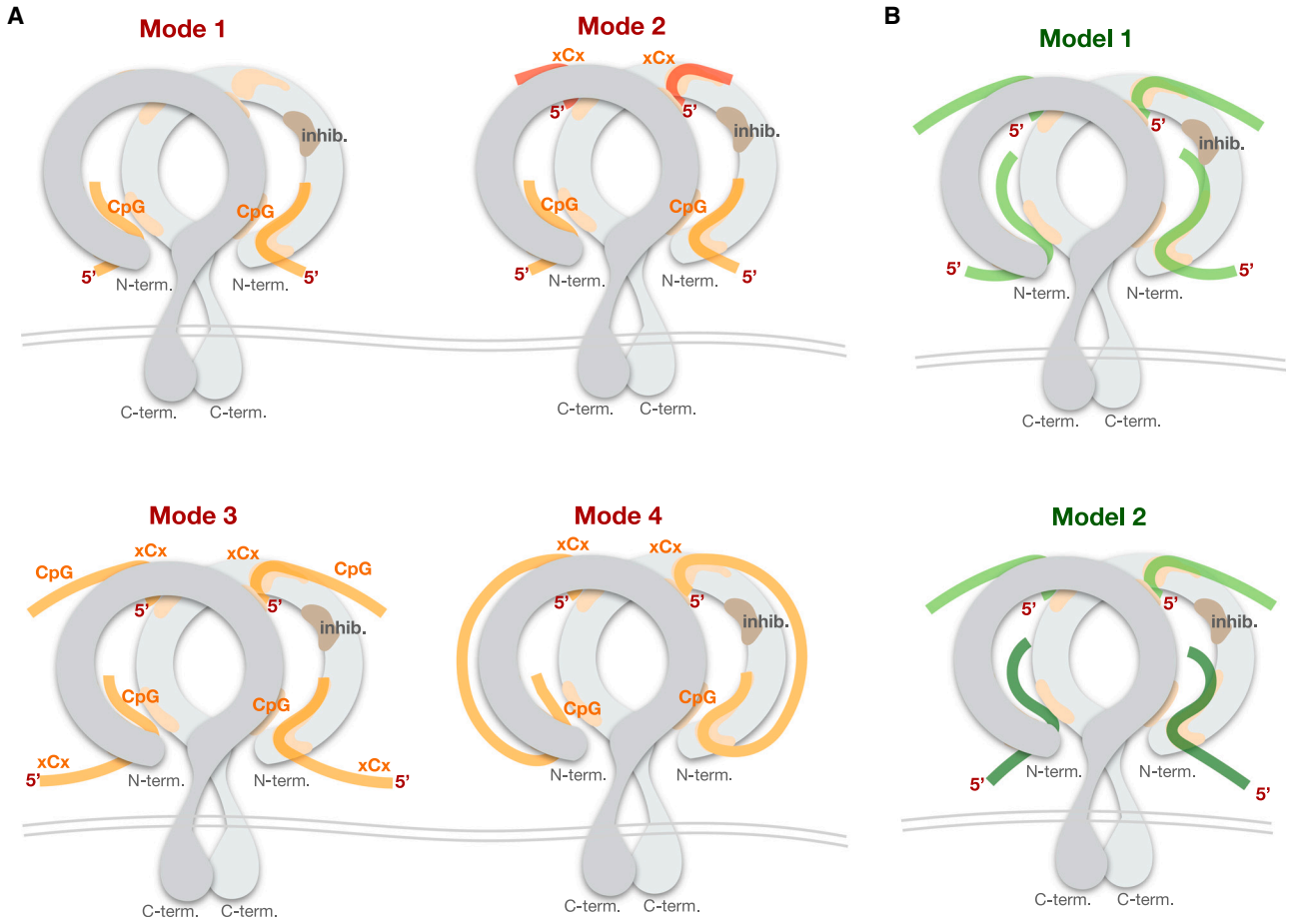


**Figure 1. Modes of oligodeoxynucleotide binding to TLR9**

The binding of its cognate ligand single-stranded DNA to TLR9 leads to a confirmational change resulting in dimerization and activation. Two distinct binding sites have been described: one binding a sequence motif containing an unmethylated CpG dinucleotide (CpG) and the other binding to a C in sequence context (xCx). A proposed inhibitory binding motif is shown in gray-orange. (A) Based on the binding to the CpG and the xCx binding site, four modes of single-stranded oligodeoxynucleotide (ODN, orange) binding have been proposed.[8,9] In mode 1, one ODN (light orange) binds to the CpG site but not the xCx site, resulting in weak activation of TLR9. In mode 2, one ODN type (light orange) binds to the CpG site and another ODN type (dark orange) to the xCx site, leading to strong activation of TLR9. In mode 3, one ODN type (light orange) exhibits two separate motifs, and one molecule of this ODN binds to the CpG site, and another molecule of this ODN type binds to the xCx motif, again leading to strong activation of TLR9. In mode 4, one ODN type (light orange) exhibits two separate motifs, and its length and structure allow one molecule of this ODN type to simultaneously bind to both the CpG and the xCx site of TLR9. (B) In the work by Pollak and colleagues,[1] model 1 of cooperative activation of TLR9 by two antisense oligonucleotides (light green) resembles mode 2 in (A), while model 2 of cooperative activation of TLR9 by two distinct types of antisense oligonucleotides (light and dark green) resembles mode 2 in (A).

the combination of the two ssDNA molecules leads to strong activation of TLR9, while each ssDNA by itself shows low or no activity even at high concentrations. Although non-competitive binding is demonstrated, there is no formal proof that the two ssDNA molecules bind to the specific CpG and the xCx binding sites of TLR9 published by Otoh and colleagues.[8,9] However, the potent synergistic TLR9 activation by the two non-CpG-containing ssDNAs strongly suggests the binding to the CpG and xCx binding sites. Agonistic binding of ssDNA to CpG and the xCx binding sites is known to elicit a conformational change, leading to dimerization and synergistic activation of TLR9. Since TLR7 and TLR8 are structurally closely related and exhibit two similar binding sites and comparable consequences in response to ligand binding, potent TLR9 activation as seen in response to non-CpG ssDNA pairs in Pollak et al.[1] is likely caused by binding to those two sites (CpG and xCx). Notably, inhibitory ssDNA binds TLR9 at a different binding site (Figure 1), but the sequence preference of this binding site remains poorly characterized. Consequently, the relative affinity of any given ssDNA pairs to the two activating sites (CpG and xCx) versus the inhibitory site likely determines the overall functional outcome of such combinatorial pairs of ssDNA. As a consequence, a precise prediction of the overall TLR9 activity of a given antisense oligonucleotide still remains out of reach.

Nevertheless, the identification of the cooperative binding mode adds important

information for the development of antisense oligonucleotides. Many of the non-CpG ODNs tested for TLR9 activation by Pollak and colleagues[1] are so-called "gapmers" (2′-O-ethyl/PS/2′-O-ethyl), a design frequently used in the current design of antisense oligonucleotides. Due to their nuclease stability, ODNs with this chemistry can be analyzed for cooperative binding independent of degradation products. Usually, the presence of CpG dinucleotides is avoided in antisense oligonucleotides to reduce recognition by TLR9. Except rare cases where antisense sequences unexpectedly activate TLR9 in the absence of CpG dinucleotides, non-CpG ODNs are usually weak at stimulating TLR9. However, the work by Pollak and colleagues[1] clearly demonstrates that even if an antisense ODN by itself is weak at TLR9 stimulation, in situations in which these ODNs are combined with other weak sequences, potent TLR9 activation can occur. This is not only relevant for an intended combination of antisense ODNs toward better or broader gene silencing. A cooperative effect of ssDNA may also become relevant when in addition to the antisense ODN, additional non-optimal ssDNA ligands are released *in vivo*, as for example by DNase-mediated processing of DNA from other sources (dead cells or NET formation). Therefore, the greatest impact of this work by Pollak et al.[1] for the antisense field is that each single ODN developed for clinical application should be thoroughly tested for cooperative activation of TLR9 (mode 2/model 2, Figure 1). That means that each ODN should be combined with well-defined oligonucleotide ligands designed to selectively bind to either the CpG or the xCx binding site of human TLR9 but not to both. The design of such defined partial oligonucleotide ligands for testing of cooperative binding clearly is an important next step toward sophisticated analysis of a potential cooperative TLR9 activity of antisense oligonucleotides that might become relevant in biological systems and *in vivo*. Such selective oligonucleotide ligands on their own are expected to exhibit no or weak TLR9 activity, while they carry intrinsic cooperative potential for TLR9 activation. The results of this work by Pollak and colleagues[1] clearly set the stage for such advanced testing of cooperative TLR9 agonistic activity. If an antisense ODN is demonstrated to have no cooperative activity on human TLR9, then it is not expected to exert such undesired TLR9 side effects even in diverse clinical situations *in vivo* in which ssDNA sequences with cooperative potential for TLR9 activation might be present.

On the other hand, if TLR9 activity is intended as for example in immune adjuvants, this work by Pollak and colleagues[1] is relevant since it demonstrates that two weak ODNs even in the absence of a CpG motif in principle can induce substantial activation of TLR9, even in the range of a potent CpG-C ODN (Pollak et al, Figure S6A). Therefore, two short ODNs, with the one optimized for the CpG binding site and the other for the xCx binding site, could possibly result in even higher TLR9 activation than achieved by currently established CpG oligonucleotides. Such a pair of short selective ODNs (binding mode 2) may exhibit advantages with regard to manufacturing, potential chemical stabilization, and biological properties when compared to one single, long CpG ODN activating TLR9 according to binding mode 3. It will be interesting to follow the development of CpG/xCx ODN pairs and whether they reach or even go beyond the potency of the gold standard agonist of human TLR9, CpG ODN 2006.[6]

## DECLARATION OF INTERESTS

No conflict of interest is declared.

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