Toll-Like Receptor 9 Contains Two DNA Binding Sites that Function Cooperatively to Promote Receptor Dimerization and Activation

Innate immunity is essential for host defense against microbial infections and relies on pattern recognition receptors (PRRs) such as Toll-like receptors (TLR) for pathogen recognition. TLR9 is known as a sensor of unmethylated CpG motif of DNA (CpG DNA) derived from pathogens. Structural and biochemical analyses demonstrate that TLR9 recognizes not only CpG DNA but also 5'-xCx motif containing DNA (5'-xCx DNA) and the latter binds to TLR9 in the presence of CpG DNA and promotes dimerization and activation of TLR9. The findings of this study will help understand the mechanism of TLR9 activation and the development of therapeutic agents targeting TLR9.

Toll-like receptor (TLR) recognizes molecular patterns from pathogenic microorganisms and activates an innate immune response through inducing the production of type I interferons and pro-inflammatory cytokines. TLR is a type-I transmembrane receptor and, to date, 10 TLRs have been identified in humans. Structural analyses have revealed that ectodomains of TLRs form a homo- or hetero-dimer upon binding of ligands. Members of the TLR7 subfamily, including TLR7, 8 and 9, are localized in endosome and participate in the sensing of nucleic acids derived from microbes, namely, ssRNA (TLR7/8) and ssDNA with CpG motifs (TLR9). So far, the crystal structure of TLR9 complexed with CpG DNA (2:2 complex) has been determined [1], where the two CpG DNAs are recognized by both TLR9 protomers and contribute to the TLR9 dimerization (**Fig. 1A**). A recent study has shown that DNAs containing the TCG or TCC motif at the 5' end in addition to the CpG motif activate TLR9 more effectively than DNAs with the CpG motif alone [2, 3] length, and dimerization properties of ODNs modulate their activation of TLR9. We performed a systematic investigation of the sequence motifs of B-class and C-class phosphodiester ODNs to identify the sequence properties that govern TLR9 activation. ODNs shorter than 21 nt and with the adenosine adjacent to the cytidine-guanosine (CG. Moreover, in TLR7 and TLR8, mononucleoside and ssRNA bind to distinct sites and exhibit synergistic effects in the dimerization of TLR7 and TLR8 [4, 5]. Because of functional and structural similarities among TLR7 subfamily members, there is the possibility that a second binding site also exists in TLR9.

Assuming that the second binding site of TLR9 might recognize 5'-TCG or 5'-TCC motif, a binding assay of 10-mer CpG DNA (AGGCGTTTTT) and 6-mer 5'-TCG DNA (TCGCCC) for TLR9 was conducted. ITC experiments showed that CpG DNA alone bound to TLR9 with a K_d value of 97 nM, while 5'-TCG DNA alone did not bind to TLR9. However, 5'-TCG DNA bound to TLR9 in the presence of CpG DNA with a K_d of 21 nM. Moreover, size-exclusion chromatography analysis showed that 5'-TCG DNA promotes dimerization of TLR9 in the presence of CpG DNA. Detailed analyses of the sequence specificity of the 5'-TCG DNA binding site in TLR9 using 5'-TCG DNA sequence variants revealed that DNAs with a cytosine nucleotide at the second position from the 5' end (hereafter referred to as 5'-xCx DNA) bound strongly to TLR9, and this binding participated in TLR9 dimerization [6].

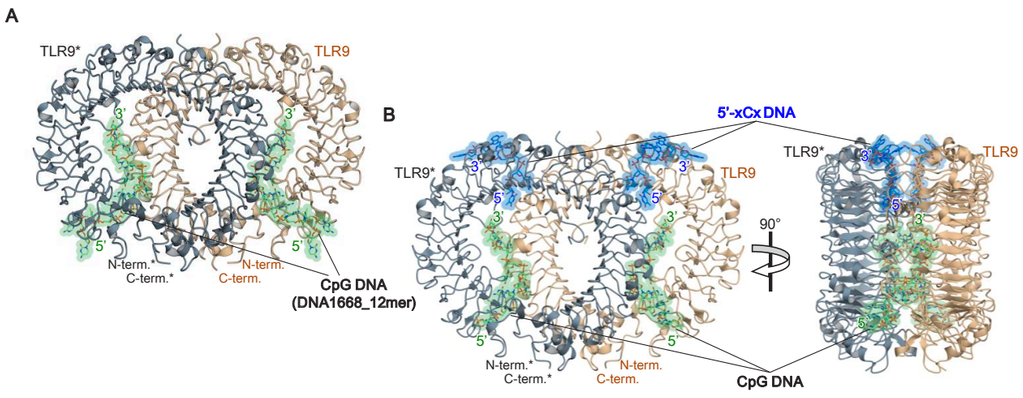

Figure 1: Activated dimer structures of TLR9. **A** Structure of horse TLR9 in complex with CpG DNA (PDB ID: 3WPC). **B** Structure of horse TLR9 in complex with CpG DNA and 5'-xCx DNA.

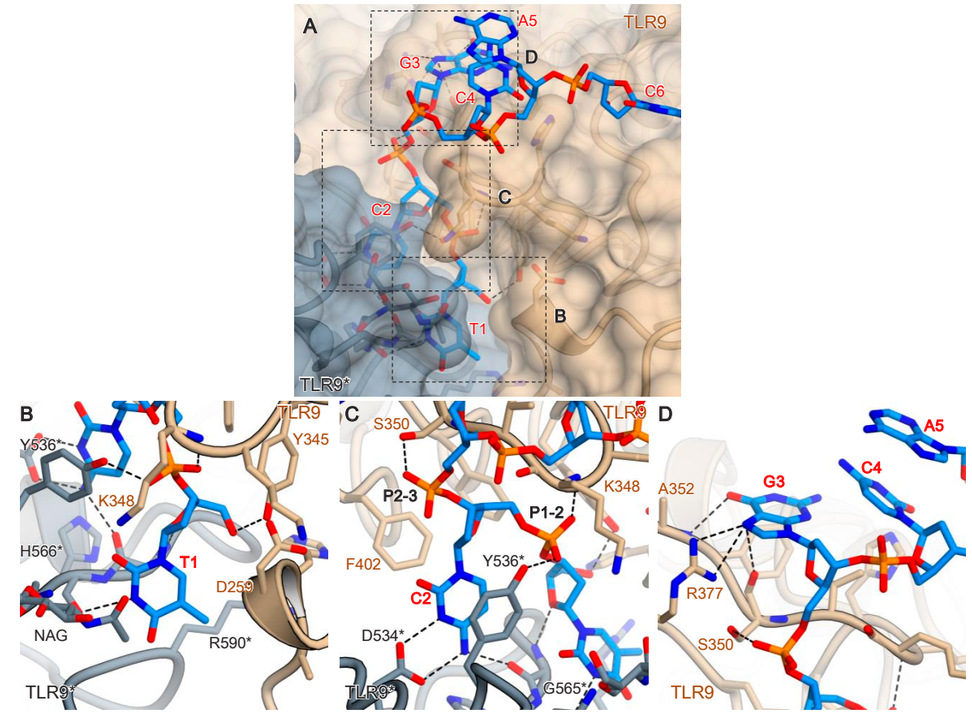

Figure 2: A Overview of 5'-xCx DNA binding site. **B-D** Close-up views of the first three nucleotide recognitions by TLR9. Hydrogen bonds are indicated with dashed lines.

To elucidate the structural mechanism by which two DNA motifs promote effective dimerization of TLR9, crystal structures of the TLR9 complexed with CpG and 5'-xCx DNA were determined using beamline AR-NE3A [6]. The structure formed a 2:2:2 complex and the overall structure was found to be essentially the same as the previously determined 2:2 complex of TLR9 and CpG DNA (**Fig. 1**). CpG DNA in the ternary complex bound to TLR9 in a manner similar to the 2:2 complex, while 5'-xCx DNA was observed in the newly identified binding site. The first three nucleotides of 5'-xCx DNA (T1, C2, G3) made multiple interactions with TLR9, which contributed mainly to the TLR9 binding and dimerization (**Fig. 2A**). The 5'-OH group of T1 was engaged with Tyr345 by hydrogen bonding and there was no space for an additional base at the 5'-end and C2 was tightly recognized by both TLR9 and TLR9* (**Fig. 2C**). These interactions determine the specificity of 5'-xCx DNA for TLR9 binding. The T1 and G3 nucleotides were loosely recognized by TLR9 (**Fig. 2B**, **2D**). These observations were consistent with the result by ITC and SEC that DNA with cytosine nucleotide at the second position from the 5'-end strongly bound to TLR9. Interestingly, C2 in the 5'-xCx DNA was located at the identical position corresponding to guanosine and uridine complexed with TLR7 and TLR8, respectively.

This study demonstrated that TLR9 senses two different types of DNAs using two distinct sites, and that such a synergistic activation mechanism of two ligands is common among the TLR7 subfamily. These structural insights into the dimerization and activation mechanism of TLR9 may contribute to drug designs for regulating TLR9 activity.

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BEAMLINE

AR-NE3A

H. Ishida, U. Ohto and T. Shimizu. (The Univ. of Tokyo)