TLR9 and the Recognition of Self and Non-Self Nucleic Acids

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ABSTRACT: Toll-like receptors (TLRs) are involved in the innate recognition of foreign material and their activation leads to both innate and adaptive immune responses directed against invading pathogens. TLR9 is intracellularly expressed in the endo-lysosomal compartments of specialized immune cells. TLR9 is activated in response to DNA, in particular DNA containing unmethylated CpG motifs that are more prevalent in microbial than mammalian DNA. By detecting foreign DNA signatures TLR9 can sense the presence of certain viruses or bacteria inside the cell and mount an immune response. However, under certain conditions, TLR9 can also recognize self-DNA and this may promote immune pathologies with uncontrolled chronic inflammation. The autoimmune disease systemic lupus erythematosis (SLE) is characterized by the presence of immune stimulatory complexes containing autoantibodies against endogenous DNA and DNA- and RNA-associated proteins. Recent evidence indicates that the autoimmune response to these complexes involves TLR9 and the related single-stranded RNA-responsive TLRs 7 and 8, and therefore some breakdown in the normal ability of these TLRs to distinguish self and foreign DNA. Evidence suggests that immune cells use several mechanisms to discriminate between stimulatory and nonstimulatory DNA; however, it appears that TLR9 itself binds rather indiscriminately to a broad range of DNAs. We therefore propose that there is an additional recognition step by which TLR9 senses differences in the structures of bound DNA.

KEYWORDS: systemic lupus erythematosus (SLE); innate immune system; toll-like receptor (TLR); TLR9; TLR7; plasmacytpoid dendritic cell (pDC); B cell; Fc receptor; B cell receptor; innate immunity; interferon (IFN); CD80; CD86; ODN; oligonucleotides (ODN); immune complexes (IC); single-stranded DNA (ssDNA); double-stranded DNA (dsDNA); single-stranded RNA; double-stranded RNA

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THE MYSTERY IN COLEY'S TOXIN

The discovery of DNA as a potent immune stimulant has a long history that is intertwined with early attempts at cancer immunotherapy. In the 1890s, Dr. William Coley, a New York surgeon, investigated the use of various live and heat-killed bacteria for the treatment of tumors, with mixed efficacy.[1,2] Today, the attenuated mycobacterium bacillus Calmette-Guerin is approved as a pharmaceutical local treatment of superficial bladder cancer.[3–5] It is well recognized that microbial material contains a wide variety of immune stimulatory substances and, not surprisingly, the search for the active ingredient in Coley's bacterial lysates was extensive. In the 1980s, Tokunaga and associates elegantly identified bacterial DNA as the principal active component of Coley's bacterial lysates.[6] This laboratory went on to show in the early 1990s that the immune stimulatory effects were inherent in bacterial—but not vertebrate—DNA and that the effects on immune cells could be mimicked by short synthetic oligonucleotides (ODN).[7,8] At the same time it became apparent that synthetic single-stranded ODN designed as antisense nucleotides could have immune stimulatory properties.[9–11] In 1995, Krieg and colleagues showed that unmethylated CG dinucleotides (CpG) are responsible for most of the immune stimulatory properties of microbial DNA or synthetic ODN.[12,13] The cytosine bases in vertebrate DNA are generally methylated and CpG sequences are found less frequently than random utilization would predict (a phenomenon that is termed *CG suppression*).[14,15] Thus, immune cells appear to be able to discriminate and respond to fine structural differences between microbial and vertebrate DNA.

Both bacterial DNA and stimulatory CpG-containing ODN can strongly activate cells of the innate immune system, including macrophages and immature dendritic cells. Cellular activation by CpG-DNA leads to antigen presentation, upregulation of MHC class II molecules, and costimulatory molecules as well as release of proinflammatory cytokines and type I interferons (IFN).[12] The antitumor activities of the early Coley bacterial extracts may have been the result of the action of these cytokines, and there is renewed interest today in the use of defined immune stimulatory ODNs as immune modulators and anticancer therapies.[16]

TLR9 IS THE PRINCIPAL SIGNALING RECEPTOR FOR CPG-DNA

Toll-like receptors (TLRs) are a family of type I transmembrane receptors that are critically involved in the recognition of conserved molecular signatures that trigger the innate immune system.[17] At least 10 different TLRs are expressed in humans. TLRs are activated by a large number of conserved

molecules associated with pathogens, suggesting that they function to alert the innate immune system to the presence of invading pathogens. However, TLRs can also be activated in some pathophysiological disease states that are associated with the presence of altered self-molecules.[18,19]

In 2000, it was shown that the receptor mediating the immune stimulatory effects of DNA is a member of the TLR family. By generating mice deficient in *tlr9* it was definitively demonstrated that TLR9 controls the cellular activation by CpG-DNA.[20] In the last decade, we have learned that TLR9 can recognize a variety of DNA sequences and that the quality and quantity of response can be dictated by the sequence and structure of DNA.[21–25] Over the last decade, several classes of stimulatory ODN based on their sequence and biological activities have been designed. Monomeric CpG-rich DNA, such as DNA sequences of the B-class (also known as D-class), can potently activate B cells and can induce maturation and production of inflammatory cytokines in plasmacytoid dendritic cells (pDCs).[22] These DNA sequences, however, lack the ability to induce type I IFNs in pDCs.[23] In contrast, A-class (also termed *K-class*) CpG-DNA can form secondary structures due to a central palindromic sequence and can assemble into nanoparticle-like complexes through the intermolecular interactions of polyG motifs that flank the central palindromic sequences.[24,26] This class of CpG-DNA can activate TLR9 to induce the production of large amounts of type I IFNs in pDCs. A third class of stimulatory CpG-DNA, the C-class CpG-DNA, combines the structural and functional features of A- and B-class. C-class ODN form hairpin structures as well as tertiary structures such as dimers and can lead to the production of type I IFNs.[21] It remains unclear how the formation of secondary structures in CpG-DNA can induce different signals from the same signaling receptor TLR9. It has been proposed that the IFN induction by A-class ODN depends on the duration and compartmentalization of ligand–receptor interaction in murine dendritic cell subtypes.[27] However, it is also conceivable that the structural CpG-DNA differences could lead to an alternative utilization of a co-receptor or a second signal from another receptor, which is required for IFN production. One should keep in mind that the different synthetic ODNs that are currently used to stimulate TLR9 have been developed to elicit a certain quantity of stimulation or quality of response. While particular CpG-DNA sequences are certainly helpful in elucidating the pharmacology of the TLR9 receptosome, the structures of naturally occurring TLR9 activators are most likely heterogeneous and differ from the synthetic CpG-DNA. For example, synthetic CpG-ODN contains phosphorothioate linkages in place of the phosphodiester linkages in naturally occurring DNA. Furthermore, the length and structure of naturally occurring DNA may differ depending on DNA source, status of enzymatic degradation, and the mode of DNA delivery. Therefore, natural microbial and endogenous DNA cannot be categorized into one of these classes of CpG-ODN and the cellular responses to DNA can differ greatly.

ENDOGENOUS DNA CAN LEAD TO INFLAMMATORY DISEASE VIA TLR9

Although recognition of DNA appears to serve to identify non-self pathogens, there is ample evidence that this ability to discriminate between foreign and self-DNA can sometimes be circumvented, leading to the development of autoimmune disease. The immune stimulatory property of DNA has recently been implicated in the pathogenesis of systemic lupus erythematosus (SLE). SLE pathogenesis involves loss of tolerance to self-antigens, which leads to the production of autoantibodies.[28,29] In sera of SLE patients autoantibodies are found against nuclear antigens, nucleic acids, and macromolecular complexes, such as chromatin or ribonucleoprotein particles. Tissue deposition of immune complexes (IC) can trigger immune-mediated tissue injury, which can lead to inflammation and injury of multiple organs.[30,31] ICs in SLE are believed to develop as part of a vicious cycle of an autoimmune response. The original trigger of autoimmunity remains elusive. However, it is clear that the disease is of a polygenic, multifactorial nature[32,33] and many of the genes that are defective in SLE patients are involved in the removal of cellular debris and nuclear material.[34–43] Thus, dysfunctional clearance of endogenous immunogenic material, such as may result from cell damage or viral infection, could trigger and/or maintain autoantibody production. The production of autoantibodies against DNA itself or against self-proteins associated with DNA results in DNA-containing ICs that are, as discussed below, capable of stimulating immune cells. SLE patients exhibit upregulation of IFN-induced genes,[44–46] normally the hallmark of viral infection, suggesting that continued stimulation by endogenous nucleic acids may be involved in the maintenance of the autoimmune state. Interestingly, the DNA that is recognized by antinuclear and anti-DNA antibodies in SLE patients has a CpG content that is similar to the composition of microbial DNA. The CpG frequency in SLE IC is 5 to 6 times higher than random DNA from the human genome and these CpG-rich complexes have been found to be immune stimulatory.[47,48]

For TLRs, the ability to selectively respond to self and non-self or altered self carries with it the inherent risk of the misinterpretation of molecular signatures. The pathogenesis of SLE may be one example of erroneous TLR activation, and studies in both mice and humans point to a role for TLR9 in this disease. Work originating from the Marshak–Rothstein group shows that rheumatoid factor autoreactive B cells can be activated by dual engagement of the antigen receptor and TLR9 (Fig. 1).[49] B cells that were non-reactive for rheumatoid factor could also sense IgG/DNA ICs, and this cellular activation required the presence of hypomethylated CpG motifs in the DNA.[50] In murine dendritic cells, chromatin-containing ICs activated cells in an Fc gamma receptor-dependent manner (Fig. 1).[51] These data imply that anti-DNA ICs can trigger systemic inflammation in a TLR9-dependent manner and suggest TLR9 as a potential target for pharmacologic intervention. Mouse *in vivo* studies, however, have

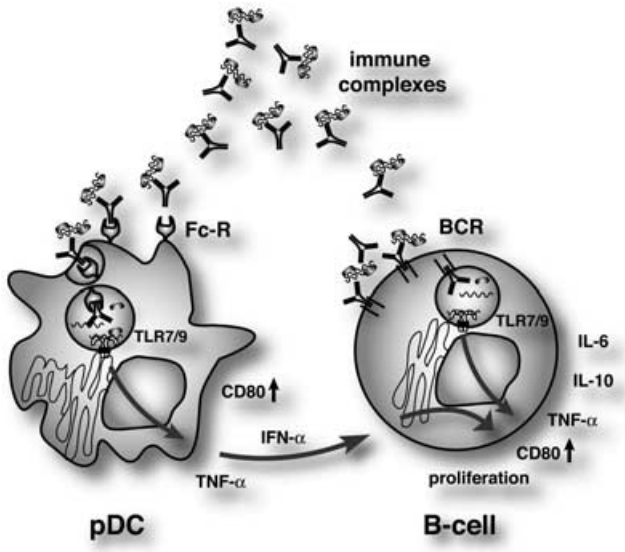

FIGURE 1. ICs containing DNA or RNA activate human pDCs and B cells via TLRs 7 and 9. DNA- or RNA-containing ICs isolated from patient sera or reconstituted *in vitro* can activate human pDCs and B cells. The uptake of ICs proceeds in pDCs mainly via Fc receptors and in B cells predominantly via the B cell receptor. Immune complex associated DNA or RNA can stimulate endosomally localized TLRs 7 or 9, which results in the activation of pDCs and B cells. PDCs are able to regulate B cell differentiation and antibody production as they can synergistically enhance B cell responses toward stimulatory ICs.

not produced a clear picture of the role of TLR9 in the initiation or progression of DNA-IC-associated diseases. While two studies have shown that lack of TLR9 in SLE-prone MRL/lpr mice resulted in inhibition of autoantibody production,[52,53] another study found unchanged autoantibody titers and increased immune pathology in mice of the same MRL/lpr background lacking *tlr9*.[54] This latter finding was partially confirmed in a second SLE-like mouse model involving *tlr9−/−* mice expressing a mutant form of phospholipase Cg2.[55] Yet, MRL/lpr mice deficient in TLR9 and TLR7 signaling due to knockout of the TLR adaptor molecule, MyD88, show decreased levels of chromatin-, Sm-, and rheumatoid factor-specific autoantibody titers.[56] Furthermore, this study also indicated that TLR7 recognizes RNA-containing IC and activates autoreactive B cells. Therefore, compensatory mechanisms between TLRs could explain the conflicting results regarding the *in vivo* role of TLR9 in IC-associated diseases. Thus, the role of TLRs 7 and 9 in disease should be tested together in autoimmune-prone mice.

In humans, IFN production by pDCs can be stimulated by small nuclear ribonucleoprotein particles, such as the prototypic autoantigen U1 snRNP, via TLR7 as well as by SLE IC via TLR9.[57,58] The immune stimulatory

activity of circulating DNA- and RNA-containing IC requires uptake into intracellular compartments via Fc receptors. Thus, TLR9 recognition of DNA and TLR 7 and 8 recognition of RNA may both be important in human disease by mediating hyper-immune responses to DNA- or RNA-containing ICs (Fig. 1).

TLR9 RECOGNIZES DNA IN ENDO-LYSOSOMAL COMPARTMENTS

TLRs that primarily recognize microbial cell wall constituents, such TLRs 1, 2, 4, 5, and 6, are found on the surface of immune cells.[59–63] In contrast, TLRs 3, 7, 8, and 9 are localized to endosome and lysosome compartments inside the cell.[64–68] This group of TLRs presumably evolved to recognize nucleic acids of pathogens that have been endocytosed or phagocytosed, or viruses that have invaded the cell. Each of these TLRs is activated by different nucleic acid structures or related molecules: TLR3 is stimulated by double-stranded RNA,[69] TLRs 7 and 8 by single-stranded RNA and imidazoquinolines,[70–74] and TLR9 by single-stranded DNA.[20]

The mode of activation of these intracellular TLRs is complex and involves several indispensable steps. The first event in the recognition of immune stimulatory DNA by TLR9 is the uptake of DNA by the cells (FIG. 2). The uptake mechanism is also one of the least well-understood steps in this process. Uptake varies depending on the structure of the DNA. For example, many cell types can internalize single-stranded DNA. In contrast, double-stranded DNA enters most cells only poorly, although the uptake can be greatly enhanced by

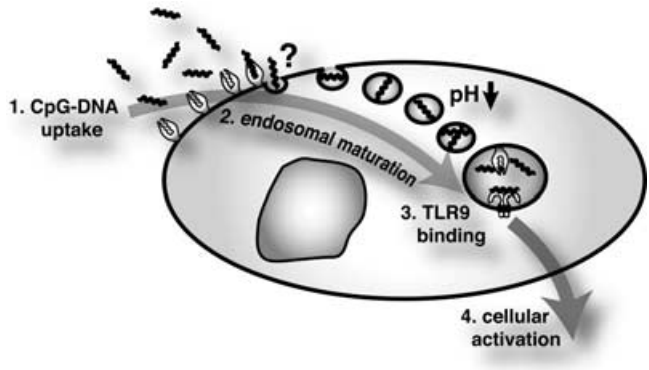

FIGURE 2. TLR9 activation proceeds in a multi-step process. Many cells can bind to and efficiently take up single-stranded DNA in a receptor-mediated pathway. The surface receptor has not been clearly identified (1). In a process termed *endosomal maturation* (2) endosomes are acidified and binding to TLR9 takes place (3). The binding of CpG-TLR9 results in the initiation of signaling ultimately leading to various aspects of cellular activation (4).

complexing DNA with cationic lipids. Similarly, most cells do not efficiently take up single-stranded RNA, but cationic lipids can also facilitate uptake, as well as stimulation of TLRs 7 and 8. Double-stranded RNA enters cells with modest efficiency, and its efficacy is also enhanced by cationic lipids or, more classically, by mixture with DEAE-dextran. A number of studies have examined uptake of single-stranded DNA, particularly in the context of antisense nucleotide research. Single-stranded DNA appears to enter cells by receptor-mediated endocytosis.[75–79] A number of cell-surface proteins have been described that may mediate uptake of single-stranded DNA including scavenger receptors,[80] CD11b/CD18,[81] and a variety of uncharacterized binding proteins.[75,78,82,83] However, conclusive evidence for a particular single-stranded DNA receptor has yet to be demonstrated (FIG. 2). Uptake can be bypassed by the use of cationic lipids, and many nonstimulatory DNAs become stimulatory when introduced via this method, suggesting that selectivity is in part determined by uptake.[84]

The subsequent step in signaling to nucleic acids, endosomal maturation, is also an imprecisely defined process (FIG. 2). Drugs that interfere with endosomal acidification, such as bafilomycin, can block signaling induced by nucleic acids or homologous molecules, suggesting that a low pH environment is required for signaling.[85] The antimalarial drug, chloroquine, can raise the pH in the endosomal microenvironment and is also a potent TLR9 inhibitor. However, early studies on chloroquine have also demonstrated that it can bind DNA and thereby could theoretically also impair the recognition of stimulatory DNA.[86] There is experimental evidence that DNA binding to TLR9 has an optimal pH between 5 and 6.[87] This finding suggests that there are regulatory mechanisms that only allow TLR9 activation in certain pH-defined subsets of endosomes and prevent activation of TLR9 in other subsets or outside endosomes. Additionally, DNA is likely to be highly concentrated within the endosome, as is the case for other endosomally targeted molecules, and the concentration of DNA could thus influence how and which DNA sequences can activate TLR9.[84] The significance of the endosomal location of DNA recognition is underlined by a study showing that TLR9 engineered to localize to the cell surface can respond to self-DNA and fails to respond to viral DNA.[88] Similarly, immune complex uptake is facilitated by the B cell receptor and/or Fc receptors and IC-associated nucleic acids are only stimulatory after uptake.[57,58]

MECHANISM OF TLR9 ACTIVATION

The molecular mechanisms leading to the activation TLR9 by DNA are not fully understood. Several studies and methodologies have indicated that TLR9 can directly bind CpG-DNA. The consensus now emerging is that DNA binding to TLR9 does not seem to determine functional outcome, since both stimulatory and nonstimulatory DNA sequences are shown to bind TLR9.[65,89] These

findings are supported by the fact that nonstimulatory (control) CpG-DNA can competitively block TLR9 activation by stimulatory DNA sequences and compete for binding at the level of TLR9.[65] Potent TLR9-inhibiting sequences, so-called inhibitory ODN, have been described. These so-called inhibitory ODN are approximately 15 bases long, have a phosphorothioate backbone, and contain two stretches of sequences that influence their inhibitory function: a pyrimidine-rich triplet, preferably CCT, which is positioned with a spacer 5' to a GGG sequence.[90–92] It is likely that these oligonucleotides act as direct TLR9 antagonists that can efficiently compete with stimulatory DNA for binding, yet not trigger signaling. Along this line, our own unpublished work using a homogenous binding assay indicates that crude sequence specificity can be observed when comparing homopolymers. For example, poly-adenine (poly-A) shows low affinity binding whereas poly-T binds with higher affinity. An important criterion for both binding to and signaling via TLR seems to be the length of the ODN and/or the degree to which the ODN are clustered. Collectively, these results suggest that differences in ligand binding may not entirely explain the discrimination between stimulatory and nonstimulatory DNAs. Rather, TLR9 may bind to a broad range of nucleic acids, but only respond to a subset of these, suggesting a signaling event that is subsequent to, and distinct from, DNA binding. This event could be a receptor rearrangement after binding to stimulatory sequences or the recruitment of a cofactor that associates with TLR9 and provides a sequence-specific signaling function. Although a cofactor that mediates sequence-specific signaling cannot be ruled out, a more compelling explanation is that TLR9 itself possesses both a sequence non-specific binding function and a motif-specific signaling function. For example, there may be multiple sites of DNA interaction on TLR9, as was suggested for TLR3 and double-stranded RNA.[93] In this latter scenario, stimulatory ODN would coordinate and/or rotate the extracellular domain(s) of TLR9 in a way that orients the signaling domains in a signaling-competent manner. Together, our understanding of the molecular events in TLR9 function is still fragmentary and hypothetical. It remains of pivotal importance to define the exact molecular mechanisms of TLR9 activation, as this will facilitate the development of TLR9-modulating therapies for a broad range of immune pathologies.

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