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# Toll-like Receptor 9 and Autoimmunity

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## 1. Introduction

Most studies that have explored the molecular and cellular basis to the generation of systemic autoimmune diseases have focused on the role played by the specific immune system. This is hardly surprising since systemic autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are associated with the production of autoantibodies. The latter include rheumatoid factor (RF), an autoantibody that is directed toward normal antibodies, antinuclear antibodies, and antibodies to DNA. However, in the last 2 years the innate immune system has jumped to the fore as a key contributor to these diseases. This has been due mainly to a pioneering report showing the key role played by the innate immune system in promoting the production of RF in a manner independent of help from T cells (Leadbetter *et al.*, 2002). This process is facilitated by a member of the toll-like receptor (TLR) family, namely TLR9. This chapter initially overviews the crucial involvement of TLR9 in mediating the immunostimulatory effects of bacterial DNA. It then describes a role for TLR9 in recognizing self-DNA leading to the activation of B cells and production of autoantibodies. The intracellular signaling pathway employed by TLR9 is also discussed and its value as a therapeutic target for the design of novel strategies for treating autoimmune diseases is emphasized.

## 2. TLRs as Receptors for Pathogen-Associated Molecules

Human TLRs play crucial roles at the host–pathogen interface due to their capacity to recognize pathogen-associated molecules. Many of the TLRs have defined functions in the innate immune system. Thus TLR2 recognizes peptidoglycan and bacterial lipoprotein from Gram-positive bacteria (Aliprantis *et al.*, 1999; Takeuchi *et al.*, 1999), TLR3 mediates responses to double-stranded RNA (Alexopoulou *et al.*, 2001), TLR4 is involved in recognition of Gram-negative

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lipopolysaccharide (LPS) (Poltorak *et al.*, 1998; Chow *et al.*, 1999; Hoshino *et al.*, 1999; Qureshi *et al.*, 1999; Takeuchi *et al.*, 1999), TLR5 recognizes bacterial flagellin (Hayashi *et al.*, 2001), TLR7 and TLR8 sense single-stranded RNA (Diebold *et al.*, 2004; Heil *et al.*, 2004), and TLR9 functions as a receptor for bacterial DNA (Hemmi *et al.*, 2000). Some TLRs, such as TLR2 and TLR6, also show functional cooperativity (Ozinsky *et al.*, 2000). The engagement of TLRs by pathogenic components results in induction of specific gene expression profiles that are suited to ensuring efficient removal and destruction of the invading pathogen.

### 3. TLR9 and the Immunostimulatory Effects of Bacterial DNA

TLR9 acts as a recognition system for bacterial DNA containing unmethylated CpG motifs. Since these motifs are quite rare and predominantly methylated in vertebrate DNA, TLR9 provides a means to distinguish between self and non-self. The expression of TLR9 in humans is mainly restricted to plasmacytoid dendritic cells (Krug *et al.*, 2001) and B cells (Bauer *et al.*, 2001). Its engagement in dendritic cells by unmethylated bacterial CpG motifs synergizes with CD40 ligand to induce high levels of IL-12 that facilitates activation of Th1 cells (Krug *et al.*, 2001). In addition, CpG motifs induce high-level expression of costimulatory molecules in plasmacytoid dendritic cells and this ensures strong activation of allogeneic T cells (Hartmann *et al.*, 1999).

CpG motifs are extremely strong stimulators for B cells, activating them to enter the G1 phase of the cell cycle (Krieg *et al.*, 1995). Indeed, relatively low concentrations of CpG DNA can synergize with the B cell receptor (BCR) and cause a 10-fold increase in B cell proliferation and antigen-specific antibody secretion. Furthermore, CpG DNA can produce antiapoptotic effects in B cells. Thus CpG DNA can inhibit the proapoptotic capacity of BCR ligation in B cell lines (Yi and Krieg, 1998a) and can promote survival of primary B cells in culture by blocking their spontaneous apoptosis (Yi *et al.*, 1998a). The ability of CpG DNA to promote sustained activation of the prosurvival transcription factor NF $\kappa$ B is a major contributor to these antiapoptotic effects (Yi *et al.*, 1998a, 1999; Yi and Krieg, 1998a). In addition to enhancing survival and proliferation of B cells and facilitating increased secretion of antigen-specific antibodies, CpG DNA can also induce the expression of costimulatory molecules in B cells (Krieg *et al.*, 1995) and thus increase their efficacy with respect to antigen presentation and T cell activation. Since the induction of many of these costimulatory molecules is dependent on NF $\kappa$ B (Medzhitov *et al.*, 1997), this transcription factor emerges as a key player in mediating the biological effects of CpG DNA. Consequently, the intracellular signaling pathways employed by TLR9 in mediating activation of NF $\kappa$ B in response to CpG DNA has become the focus for much research.

### 4. TLR9 and Intracellular signaling

Cells display DNA-binding proteins on their surface, but lack selectivity in recognizing specific sequences (Krieg *et al.*, 1995). This study also demonstrated that cell uptake of CpG DNA is required to produce its effects in B cells. While

the mechanism of uptake is incompletely understood, internalized CpG DNA has been localized to the endosome and studies suggest that this is the site where intracellular signaling pathways are initiated by the DNA. Thus agents such as chloroquine, which interfere with endosomal acidification and/or maturation, block the signaling pathways (Hacker *et al.*, 1998; Yi and Krieg, 1998b; Bauer *et al.*, 2001) and immunostimulatory effects (Yi *et al.*, 1998b) of CpG DNA. In contrast, other pathogen-associated molecules such as LPS, which are recognized by specific cell surface receptors, are unaffected by chloroquine (Yi *et al.*, 1998b). TLR9 was subsequently shown to be the specific receptor for CpG DNA since the dendritic and B cells of mice genetically deficient in TLR9 are unresponsive to CpG (Hemmi *et al.*, 2000). Furthermore, some evidence suggests that TLR9 is localized to the endosomes where it may be able to physically interact with the internalized CpG DNA (Hemmi *et al.*, 2000; Takeshita *et al.*, 2001).

The proximal signaling events subsequent to TLR9 engagement by CpG have been well characterized. Like most other TLRs, activation of TLR9 recruits the adaptor molecule Myd88 (Medzhitov *et al.*, 1998). Indeed, CpG DNA, TLR9, and Myd88 colocalize in late endosomes and the initiation of signaling is dependent on endosome maturation (Takeshita *et al.*, 2001; Ahmad-Nejad *et al.*, 2002). Myd88 subsequently recruits and activates members of the IL-1 receptor-associated kinase (IRAK) family (Muzio *et al.*, 1997; Wesche *et al.*, 1997; Kobayashi *et al.*, 2002; Li *et al.*, 2002; Suzuki *et al.*, 2002). The IRAK-Myd88 association triggers hyperphosphorylation of IRAK by itself (Cao *et al.*, 1996) and/or by other additional kinases (Li *et al.*, 1999), leading to its dissociation from Myd88 and its interaction with and activation of the downstream adaptor TNF receptor-associated factor 6 (TRAF-6) (Burns *et al.*, 2000). The latter is a ubiquitin ligase that activates TGF $\beta$ -activating kinase (TAK1) (Ninomiya-Tsuji *et al.*, 1999). Activated TAK1 promotes downstream activation of the I $\kappa$ B-kinases (IKK), IKK $\alpha$  and IKK $\beta$ , that form a large multi-protein complex with a scaffold protein called NEMO (IKK $\gamma$ ). Of the two active IKK isoforms, IKK $\beta$  appears to be the more important for CpG signaling (Chu *et al.*, 2000). It affects phosphorylation of members of the inhibitory I $\kappa$ B family (I $\kappa$ B- $\alpha$  and I $\kappa$ B- $\beta$ ) that normally sequester NF $\kappa$ B in an inactive form in the cytosol (Yi and Krieg, 1998a). The phosphorylation of the I $\kappa$ B proteins represents a signal for polyubiquitination followed by their degradation via the 26 S proteasome and this allows for translocation of NF $\kappa$ B to the nucleus, where it activates a plethora of genes encoding proinflammatory proteins and costimulatory molecules (Medzhitov *et al.*, 1997; O'Neill, 2002). Interestingly, the activation of NF $\kappa$ B is also a requisite for CpG-induced protection of B cells against apoptosis (Yi and Krieg, 1998a; Yi *et al.*, 1999). Such activation of NF $\kappa$ B is sustained in nature and usually associated with prolonged disappearance of the inhibitory I $\kappa$ B- $\beta$  protein (Bourke *et al.*, 2000).

In addition to NF $\kappa$ B activation, CpG motifs can also engage TLR9 to stimulate mitogen-activated protein kinase (MAPK) signaling cascades and activate multiple transcription factors, including AP-1. Thus CpG DNA induces phosphorylation of p38 and c-Jun N-terminal kinase (JNK) in B cells (Yi and Krieg, 1998b). Interestingly, while CD40 utilizes many of the same signaling molecules and activates the same pathways as CpG in B cells (Brady *et al.*, 2000, 2001), the

activation of MAPKs by CpG is slower in onset but longer in duration (Yi and Krieg, 1998b). The sustained activation of both NF $\kappa$ B and MAPK pathways by CpG in B cells suggests that these intracellular pathways are long-lived in response to CpG and not subject to acute downregulation. Whilst the mechanisms by which the MAPKs are activated by TLR9 are incompletely understood, upstream regulators have been identified. Such regulators also play integral roles in mediating activation of NF $\kappa$ B. Thus TAK1 can activate the MAPKKs MKK3/6 and MKK4, which in turn activate p38 and JNK, respectively (Ninomiya-Tsuji *et al.*, 1999). The activation of the MAPK pathways by CpG motifs in B cells contributes significantly to their biological responsiveness to CpG since inhibitors of these pathways block CpG-induced secretion of cytokines by B cells (Yi and Krieg, 1998b).

The overall effects of CpG DNA on B cells are thus mediated via TLR9 activation of the NF $\kappa$ B and MAPK pathways. As stated previously, TLR9 and BCR show strong functional synergy in activating B cells. It is worth noting that a recent report has provided a molecular basis for this synergy by demonstrating that their signaling pathways converge at the level of NF $\kappa$ B and p38/JNK (Yi *et al.*, 2003).

## 5. CpG Sequences in Self-DNA Trigger Autoantibody Production

For many years vertebrate self-DNA was considered to be immunologically inert. However, a report in 2002 demonstrated that immune complexes containing self-DNA activate RF-specific B cells (Leadbetter *et al.*, 2002). This study employed a genetically engineered mouse strain in which most B cells express a BCR with low affinity for self-IgG2a. The affinity is insufficient to trigger activation of the B cells. However, other workers had previously shown that when the mice are crossed with a strain susceptible to autoimmune diseases, such as RA and SLE, the self-IgG2a becomes a powerful activator of B cells and stimulates B cell proliferation and secretion of high levels of circulating RF autoantibodies (Wang and Shlomchik, 1999). Leadbetter *et al.* (2002) went on to show that self-IgG2a accumulates in complexes with self-DNA in the bloodstream of the autoimmune mice. It is likely that the self-DNA is released during physiological and/or pathological cell death. In this model the synergy between the signals originating from BCR activation by self-IgG2a and TLR9 activation by self-DNA is sufficiently powerful to provoke strong B cell activation. Furthermore, the BCR is likely to facilitate the uptake of self-DNA and its ultimate delivery to endosomal TLR9 (Viglianti *et al.*, 2003). However, as stated previously, TLR9 acts as a receptor for DNA containing unmethylated CpG motifs, and mammalian DNA tends to be methylated. This questions the role of TLR9 in recognizing self-DNA. However, mammalian DNA is not exclusively methylated and indeed methylation is restricted to 70–80% of the CpG motifs (Bird, 1987). The remaining 20–30% of unmethylated CpG motifs may be responsible for self-DNA activating TLR9. This notion is supported by two findings. First, DNA methylation status is

reduced in cells from animals and humans with autoimmune disease (Richardson *et al.*, 1990). Second, drugs such as azacytidine that inhibit CpG methylation cause an autoimmune disease with features of lupus (Yung *et al.*, 1995). Krieg (2002) has put forward an alternative model wherein cross-linking of the BCR may be sufficiently extensive to reduce the specificity of TLR9 recognition, allowing its activation by self-DNA (Goeckeritz *et al.*, 1999). Interestingly, Krieg (2002) also suggests activating immune complexes may result from an earlier loss of B cell tolerance to DNA-associated self-antigens. This is consistent with the clinical finding that most autoantibodies in SLE are directed against nuclear antigens that bind DNA (Krieg, 2002). Since high levels of RF against IgG are measured in patients suffering from RA and SLE, therapeutic potential may lie in designing novel approaches to control the production of RFs. The key role played by TLR9 in promoting RF production makes this pathway an ideal target for therapeutic intervention.

## 6. TLR9 as a Target for Regulating RF Production

Since TLR9 acts as a costimulus for autoreactive B cells, and because endosomal maturation and acidification is required for its signaling, a molecular basis is now at hand to explain the therapeutic effects of chloroquine in systemic autoimmune diseases. Chloroquine interferes with endosomal acidification and maturation and blocks all CpG-TLR9 signaling pathways without inhibiting other B cell activators (Hacker *et al.*, 1998; Yi and Krieg, 1998b; Yi *et al.*, 1998b; Bauer *et al.*, 2001). Chloroquine and other similarly acting agents have now been shown to block immune complex-induced activation of autoreactive B cells (Leadbetter *et al.*, 2002) and this is likely to underlie the therapeutic efficacy of chloroquine. This highlights the value of targeting the TLR9 pathway in the design of novel therapeutics for the treatment of autoimmune diseases. Valuable clues in this regard may be provided by identifying physiological mechanisms for controlling the TLR9 pathway. A recent report has shown that continuous self-antigen signaling via the BCR MAPK pathway inhibits CpG DNA-induced plasma cell differentiation and controls TLR9-mediated autoantibody production (Rui *et al.*, 2003). It is also worth noting the presence of a number of endogenous regulators of TLR signaling pathways. SIGIRR is a membrane protein that negatively regulates TLR signaling (Wald *et al.*, 2003). Myd88S, a splice variant of Myd88, inhibits Myd88-dependent pathways (Janssens *et al.*, 2003). IRAK-M is a member of the IRAK family and acts as a negative regulator of TLR signaling pathways (Kobayashi *et al.*, 2002). Furthermore, the phosphorylation of IRAK-1 during TLR signaling ultimately leads to its degradation by proteosomes and this may represent a regulatory mechanism by which cells become desensitized after prolonged activation of TLRs (Yamin and Miller, 1997; Li *et al.*, 2000). Finally, the antiapoptotic protein A20 acts to terminate signaling pathways triggered by TLRs (O'Reilly and Moynagh, 2003; Boone *et al.*, 2004; Gon *et al.*, 2004). The existence of these numerous braking systems on TLR signaling pathways emphasizes the importance of tightly regulating such pathways. Some of these mechanisms

may offer future opportunity to develop novel strategies to dampen TLR9 signaling and to control the activity of autoreactive B cells in autoimmune disease. Interestingly, as stated previously, the temporal activation of TLR9 signaling pathways in B cells tends to be sustained and many of the above autoregulatory mechanisms may not be at play in these cells. The identification of means to promote these mechanisms may be of immense value in learning to regulate TLR9 signaling and autoantibody production in B cells.

## 7. Concluding Remarks

The discovery that TLR9 can promote the production of autoantibodies by synergistically activating B cells in conjunction with BCR raises the intriguing possibility that self-DNA is immunostimulatory in a T cell-independent manner. Furthermore, TLRs can no longer be regarded as recognition systems in the innate immune system that shows exclusive selectivity for pathogen-associated molecules. TLR9 compromises the capacity for absolute distinction between self and nonself. Our current molecular appreciation of the TLR9 signaling pathway provides hope for therapeutic manipulation and the design of novel strategies for treating autoimmune diseases.

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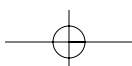
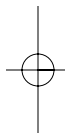
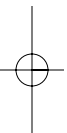
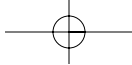
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