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TLR9 as a key receptor for the recognition of DNA ☆

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Abstract

Unmethylated DNA with CpG-motifs is recognized by Toll-like receptor 9 (TLR9) and pleiotropic immune responses are elicited. Macrophages and conventional dendritic cells (cDCs) produce proinflammatory cytokines to B/K-type CpG-DNA, whereas plasmacytoid DCs induce type I interferons to A/D-type CpG-DNA and DNA viruses. The TLR9 mediated signaling pathway is not only responsible for activation of innate immune cells, but also for mounting acquired responses. Thus, it has been attempted to exploit TLR9 ligands as a vaccine adjuvant for anti-cancer immunotherapy. Further, TLR9 mediated signaling is implicated in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus. Nevertheless, recent studies revealed that double-stranded DNA can be recognized by intracellular receptor(s) in a TLR9-independent manner. This review will focus on the roles of TLR9 in immune responses, and its signaling pathways.

Keywords: Innate immunity; CpG-DNA; Cell signaling; Type I interferon; Plasmacytoid dendritic cell; Autoimmunity

Contents

1. Introduction

Upon microbial infection, the host evokes various responses to eliminate the invading pathogens. Host immune cells elicit an innate immune response by detecting pathogen specific molecular patterns (PAMPs) and mount a strong acquired immune response as well [1]. Bacterial DNA has long been known as one of the key immunostimulatory PAMPs. The DNA fraction of *Mycobacterium bovis* BCG has been shown to be capable of activating human and mouse non-B/T cells [2]. Bacterial DNA also causes septic shock [3]. On the other hand, mammalian DNA does not induce such responses. Immunostimulatory DNA has a specific pattern of unmethylated CpG motifs (reviewed in [4]). Unmethylated

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CpG-DNA activates not only innate immunity but also acquired immunity. These motifs are not detected in mammalian DNA because most CpG sequences are methylated in mammals.

During the 1990s, several lines of accumulated evidence indicated that Toll-like receptors (TLRs) can act as pattern-recognition receptors (PRRs) that detect PAMPs. The Toll receptor was first identified in the fruit fly *Drosophila melanogaster* where it was found to be involved in an anti-fungal response [5]. Subsequently, mammalian TLRs were cloned, and their roles in the recognition of microbial components were elucidated by a mouse model with both forward and reverse genetic approaches (reviewed in [6]). TLR2 (together with TLR1 or TLR6) and TLR4 are the receptors mainly involved in the recognition of bacteria-derived ligands, such as microbial lipoproteins and LPS, respectively [7–9]. TLR3 and 7 (and possibly 8) are involved in the recognition of double-stranded (ds) and single-stranded (ss) RNA, respectively [10–13].

TLR9 was first cloned and identified as a receptor for unmethylated CpG-DNA as well as for bacterial DNA in 2000 [14]. Analysis of TLR9-deficient mice revealed that TLR9 is essential not only for pro-inflammatory cytokine production and other inflammatory responses, but it also plays a role in the induction of T helper 1 (Th1) acquired immune response and in the proliferation of B cells.

After the identification of TLR9, the mechanisms of various immune responses elicited by DNA were clarified. It has been shown that several DNA viruses are recognized and are able to elicit antiviral responses through TLR9. The TLR9-mediated recognition of DNA viruses and CpG-containing oligodeoxynucleotides (CpG-ODNs) induces type I interferon (IFN) production in plasmacytoid dendritic cells (pDCs) [15–17]. TLR7 is also highly expressed on pDCs and stimulation with ssRNA as well as RNA viruses produced vast amount of type I IFNs via TLR7.

Nevertheless, recent studies revealed that cytosolic PRRs called Retinoic acid-inducible gene (RIG-I)-like helicases (RLHs) can recognize viral RNA in the cytosol [18]. RIG-I and Melanoma differentiation-associated gene 5 (MDA5) consist of two caspase recruitment domains (CARDs) and a RNA helicase moiety. The recognition of viral RNAs by these helicases induces production of type I IFNs and an associated antiviral response. Double-stranded DNA without the CpG motif may also be recognized by cytoplasmic PRR(s) [19]. In this review, we will discuss the current understanding of the role of TLR9 in immunity from several points of view, and outline what is still not yet known.

2. The TLR9 ligands

The TLR9 ligands, CpG-ODNs, are classified into two different subtypes, B/K-type and A/D-type [20]. The B/K-type CpG-ODNs are phosphorothioate-modified throughout the sequence and are known to induce DC maturation and B cell proliferation. On the other hand, A/D-type CpG-ODNs, which are characterized by a phosphodiester backbone CpG motif and phosphorothioate-modified poly G stretches at the 5′ and 3′ ends, induce type I IFNs in pDCs [21].

TLR9 also recognizes bacterial and viral DNA. Upon *Mycobacterium tuberculosis* infection, TLR9 cooperates with TLR2 to induce innate and adaptive immune responses against the bacterium [22]. TLR9 also plays an important role in the fight against infection with *Brucella* [23] and *Streptococcus pneumoniae* [24]. Polymorphisms in TLR9 have been reported to be associated with an increased susceptibility for *Helicobacter piloli* infection, suggesting that TLR9 is involved in recognition and clearance of *Helicobacter* [25]. Genomes of DNA viruses often have unmethylated CpG motifs, and therefore act as a ligand for TLR9, and consequently DNA viruses may elicit TLR9-mediated antiviral responses. Mouse cytomegalovirus (MCMV) [15], herpes simplex virus (HSV) type 1 [16] and type 2 [17], and adenovirus [26] are recognized by TLR9 on pDC, and the resulting activated pDC produce IFN-α and other cytokines.

In addition to DNA, hemozoin (HZ) derived from the malaria parasite *Plasmodium* acts as a TLR9 ligand [27]. HZ is a metabolic product of *Plasmodium* and is known as the malaria pigment. *Plasmodium* metabolizes hemoglobin in the host red blood cells, and the resulting digested heme forms the hydrophobic polymer, called HZ. However, a recent study argued that the activation of immune cells via HZ was dependent on the presence of malarial DNA which acts as a TLR9 ligand [28]. Although the reason for this discrepancy has not been clarified yet, the cytokine-inducing activity of our HZ preparation was DNase resistant, supporting that cytokine production caused by HZ was not due to the contamination of DNA in the preparation. Of note, there is evidence that TLR9 together with TLR2 is involved in the pathogenesis of cerebral malaria [29].

In contrast to these findings, several reports have shown that DNA without CpG motifs can be a biologically active ligand for TLR9. ODNs containing nucleotide derivatives or the phosphorothioate backbone along with a bicyclic heterobase structure have immuostimulatory activity through TLR9 [30–32]. Thus, TLR9 recognizes not just CpG motifs, but DNA itself with certain structures.

3. TLR9 signaling

TLR consists of leucine-rich repeats (LRRs), a transmembrane domain and a cytoplasmic Toll/interleukin-1 receptor homology (TIR) domain. TLR9 is localized at the intracellular membrane compartment, such as the endoplasmic reticulum (ER), the endosome, and the lysosome [33]. The LRR of TLR9 is on the inside of the membrane compartment, while the TIR domain is located on the cytosolic side. Stimulation of TLR9 with its ligands leads to activation of various transcription factors, including nuclear factor κB (NF-κB) and activator protein-1 (AP-1) (Fig. 1).

TLRs transmit their signals through a specific interaction with adaptor molecules at their cytosolic TIR domain. MyD88, one of these adaptors containing a TIR domain and a death domain (DD), is known to be essential for initiating TLR9 signaling. MyD88-deficiency abolishes the activation of transcription factors and cytokine production elicited by CpG-ODN through TLR9 [34]. In contrast to TLR2, TLR3 and TLR4, other TIR domain containing adaptors, such as TRIF,

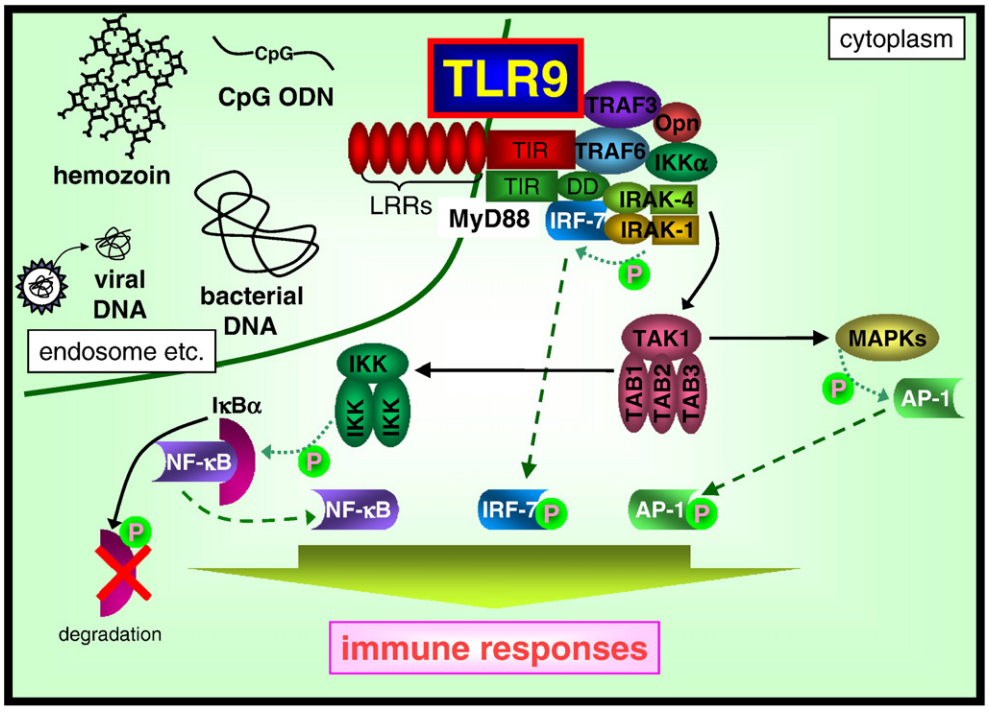

Fig. 1. An overview of the TLR9-mediated signaling pathway. TLR9 with its ligands, such CpG-oligodeoxynucleotide (ODN), bacterial and viral DNA, and hemozin, ligate mainly exist in endosomal compartments. The cytosolic TIR domain of TLR9 recruits the adaptor molecule MyD88 and other signaling molecules such as IRAK-4, and TRAF6 that are required for the signaling complex. Some transcription factors such as IRF-1, IRF-5 (not shown in this figure), and IRF-7 are also recruited to the complex and activated (described in the text). The complex in turn activates other signaling cascades that lead to the activation of NF-κB and AP-1. These activated transcription factors induce diverse immunity-related genes.

TIRAP and TRAM, are not involved in TLR9 responsiveness [35].

MyD88 in turn interacts with interleukin-1 receptor-associated kinase-1 (IRAK-1) and IRAK-4 through its DD. IRAK-4 and its kinase activity are shown to be essential for TLR9-mediated cytokine production [36]. IRAK-1 is a known substrate of IRAK-4, and phosphorylated IRAK-1 upregulates its kinase activity, and subsequently recruits tumor necrosis factor associated factor 6 (TRAF6). TRAF6 is a E3 ubiquitin ligase that catalyzes K63 type polyubiquitination together with UBC13 and Uev1A [37]. Downstream of TRAF6, transforming growth factor-β associated kinase 1 (TAK1) is activated to the phosphorylate IκB kinase (IKK) complex, which phosphorylates IκB to induce nuclear translocation of NF-κB [38]. A study of Ubc13-deificient mice revealed that UBC13 is prerequisite for the activation of mitogen-activated protein (MAP) kinases, but is dispensable for NF-κB activation, whereas TAK1 is required for both [39,40]. Transcription factors NF-κB and AP-1 are responsible for the transcription of pro-inflammatory cytokine genes, including TNF-α, IL-6 and IL-12. For the MyD88-dependent cytokine gene expression, another transcription factor, IFN-regulatory factor-5 (IRF-5) is reported to be required [41]. IRF-5 interacts with MyD88 and TLR stimulation induces nuclear translocation of IRF-5 which regulates the production of several cytokines such as IL-6, IL-12, and TNF-α.

TLR9 is originally localized in the ER, and then migrates to the endosome when the cells are stimulated [33]. Thus, TLR9 responds to the non-self DNA in the intracellular membrane compartment. Several reports have shown that treatment of cells with type III phosphoinositide 3 kinase (PI3 K) inhibitor abolished TLR9 signaling [42–44]. Because PI3 K is reported to play a role in the uptake of CpG-ODN rather than participate as a direct signaling component of the TLR9 cascade, access of DNA to the endosomal compartment might be important for signaling [45]. After the translocation of the ligands, endosomal acidification is reported to be critical for the signaling of TLR9, suggesting that localization of CpG-ODN in the endosome is a prerequisite for TLR9-mediated recognition [46,47]. However, another report showed that TLR9 enforced to express on the plasma membrane by exchanging the membrane spanning domain with that of TLR4, recognized self DNA and consequently elicited immune responses [48]. In this regard, localization of TLR9 at the intracellular membrane compartment may be critical for preventing occasional TLR9 signaling and the consequent immune response elicited by self DNAs. A mouse forward genetics screening identified Unc93B as a molecule essential for TLR3, TLR7 and TLR9 signaling. This protein is known to interact with a membrane spanning domain of TLR9. A *3D* mutation in the *Unc93b* gene impaired TLR9-mediated signaling without affecting localization of TLR9 [49] implying that the membrane spanning domain of TLR9 plays a role not only in localization, but also in TLR9 signaling itself.

4. Cell types utilizing TLR9

TLR9 is expressed on various cell types (Fig. 2). Upon B/K-type CpG-DNA stimulation, cDCs and macrophages produce proinflammatory cytokines, such as TNF-α, IL-6 and IL-12, and upregulate surface expression of MHC class II and co-

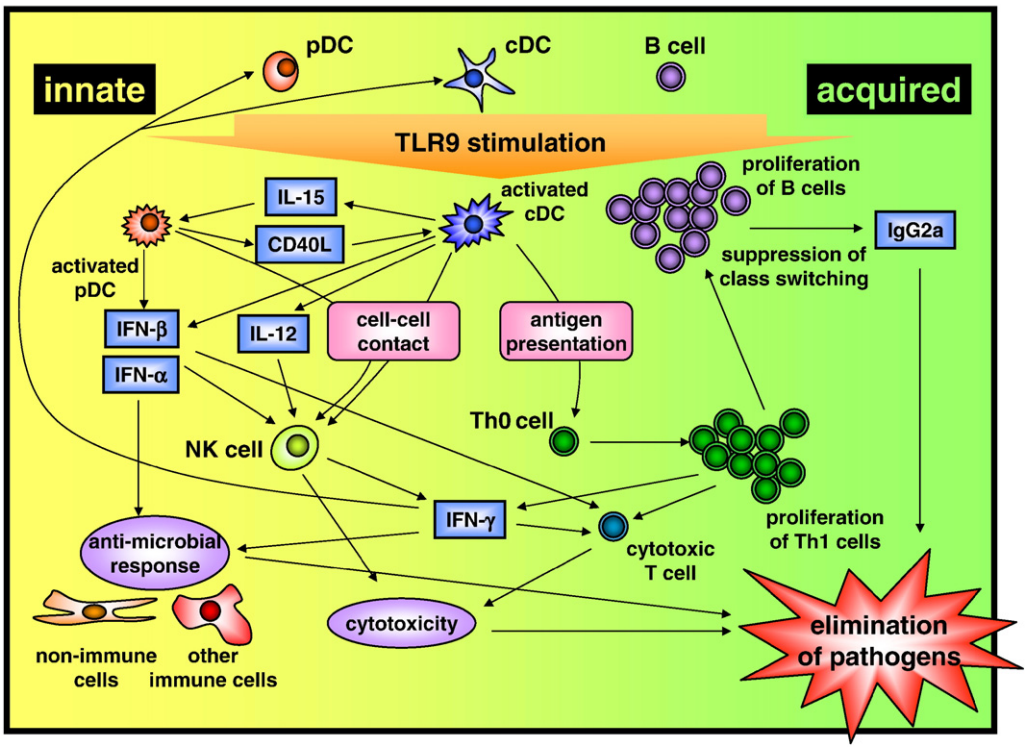

Fig. 2. Impact of TLR9 on the host immune response. The TLR9 signaling targets pDC, cDC, and B cells. IKDC (not shown in this figure) is also activated by TLR9 signaling, though the exact response from this population is still controversial. DCs produce various cytokines in cooperation. The cytokines act on various cell types, including NK cells, T cells, other immune and non-immune cells. Activated NK cells and T cells produce IFN-γ, which in turn activates DCs and others. They exhibit a cytotoxic response to eliminate virally infected cells or others. These cells also activate B cells, DCs and other immune and non-immune cells by humoral factors or cell–cell contact. TLR9 signaling directly acts on B cells to start proliferation.

stimulatory molecules [21]. DCs maturated via TLR9 act on T cells to mount an acquired immune response [14]. Indeed, CpG-ODNs are known as very strong adjuvants that polarize helper T cell responses to Th1 [20].

TLR9 is highly expressed on human and mouse pDC, a cell type identified as a "professional IFN-producing cell" that makes large amounts of type I IFNs upon contact with various stimuli including CpG-ODN (reviewed in [50]). Type I IFNs from TLR9-stimulated pDCs directly activate cytotoxic T cells [51] and helper T cells [52]. Another cell type that exhibits surface phenotypes ($B220^+CD11c^+NK1.1^+CD49b^+$) on both pDC ($B220^+CD11c^+$) and NK cells ($NK1.1^+CD49b^+$) was identified and named the IFN-producing natural killer dendritic cell (IKDC) [53,54]. However, recent studies indicated that this population of cells is rather similar to NK cells and have neither antigen-presenting activity nor type I IFN producing activity [55–59], thus the exact character of this population is still controversial.

IL-12 production to TLR9 stimulation involves interaction between pDC and cDC [60]. cDC produces IL-15 upon TLR9 stimulation. IL-15 in turn activates pDC to express CD40L on its surface. At the same time, TLR9 signaling facilitates expression of CD40 on cDC. Consequently, crosstalk between cDC and pDC via CD40-CD40L ligation leads to production of IL-12 from cDC.

Several studies indicate that TLR9-activated DCs act on natural killer (NK) cells to elicit NK cell-mediated immune responses such as cytotoxicity and production of IFN-γ. This stimulation seems to include not only paracrine activation with type I IFNs, IL-12, and IL-15, but also cell–cell contact [61]. The mode of MCMV-induced activation of NK cells is well characterized. The activation was impaired in TLR9-deficient as well as MyD88-deficient mice and the cytokine production was abolished in pDC-depleted mice, suggesting that the NK activation requires TLR9 signaling in pDC and the resulting production of type I IFN and IL-12 from pDC [15]. Other DNA viruses such as the vaccinia virus, also induces activation of NK cells [61]. This response was severely impaired in $CD11c^{high}$ DC-depleted mice, indicating that the response requires TLR9 only on cDC. These results suggest that NK cells are *trans*-activated by TLR9 on DCs. Activated NK cells produce IFN-γ, which in turn activates DCs and other immune cells. Thus, TLR9 plays a pivotal role in the crosstalk between DCs and NK cells to achieve the activation of both cell types and the resulting robust innate immune responses.

B cells also express TLR9 on their surface. B cells are directly activated through TLR9 with B/K-type CpG-ODN to directly induce proliferation [62]. The effect of CpG-ODN to facilitate B cell proliferation was found to be dependent on MyD88 and TLR9 [14]. TLR9 signaling also facilitates immunoglobulin (Ig) secretion from B cells [62]. Moreover, TLR9 stimulation directly suppresses a class switch from IgM and IgG2a to allergic IgG1 and IgE [63,64]. Interestingly, Th1 cells also inhibit this class switch. Given that Th1 cells are activated by TLR9 signaling as described above, TLR9 again plays a pivotal role in the activation of B cells and the suppression of allergic

Th2 immune responses. A study indicated that TLR signaling directly controls antibody production from B cells in collaboration with Th cells [65]. However, this finding was questioned by a more recent report [66], thus the direct contribution of TLR signaling to antibody production by B cells is still controversial.

5. TLR9-induced type I IFN production in pDCs

Type I IFNs, comprised of multiple IFN-αs and single IFN-β, are a critical part of cytokine antiviral immunity [67]. They act on cells infected with viruses to induce apoptotic cell death, they potentiate antiviral activity to surrounding cells, and they also play an important role in the development of adaptive immune responses.

pDCs are known to highly express TLR9 and TLR7, and are capable of producing large amounts of type I IFNs upon stimulation with A/D-type CpG-DNA and ssRNA [11,12,21]. On the contrary, stimulation of cDCs with the same stimulants failed to induce production of IFN-α, although small amounts of IFN-β were observed in cDCs [21].

Extensive studies have clarified the specialized molecular mechanisms of how pDC evokes type I IFN production and an antiviral response (Fig. 1). IFN-regulatory factor-7 (IRF-7) is a transcription factor that regulates expression of type I interferons (IFNs) and IFN-inducible genes [68]. IRF-7 directly interacts with MyD88 and forms a large complex with IRAK-1, IRAK-4 and TRAF6 in pDCs [69]. IRF-7 is phosphorylated in the complex and translocates into the nucleus to induce transcription of type I IFNs and IFN-inducible genes. IRAK-1-deficient cells as well as IRAK-4-deficient cells did not produce IFN-α in response to CpG-ODN [36,70]. However, IRAK-1 deficiency did not lead to the impairment of the production of other cytokines, and IRAK-1, but not IRAK-4, could phosphorylate IRF-7 *in vitro* [70]. These data suggested that IRAK-1 but not IRAK-4 acts as a kinase for IRF-7. In addition, IκB kinase-α (IKKα) is also reported to phosphorylate IRF-7 *in vitro*, and IKKα-deficient pDCs showed defective production of IFN-α in response to TLR7 and TLR9 stimulation [71]. Further studies are required for identifying the relationship between IKKα and IRAK-1. CpG-ODN-induced type I IFN production was also abrogated in the cells deficient in osteopontin (Opn) [72] and TRAF3 [73,74], whereas the production of IL-12 was not impaired in cells from these mice.

Moreover, autophagy, which is primarily known as a recycling mechanism for unnecessary cell components, is also proposed to be involved in type I IFN production from pDC in response to CpG-DNA and VSV infection [75]. Atg5 is one of proteins essential for autophagosome formation. pDC deficient in Atg5 showed defected production of type I IFNs as well as IL-12. It was shown that VSV infected pDCs to induce autophagosome formation, which looks to be essential for TLR7-mediated recognition. In addition, IFN-α, but not IL-12, production upon CpG-ODN stimulation was also impaired in Atg5-deificient pDCs. Although finite details underlining this phenomenon are yet to be clarified, it is possible that autophagy is critical for the activation of signaling pathways activated by TLR9 for inducing type I IFNs.

Stimulation of cDCs with TLR9 also activates expression of IFN-β and IFN-inducible genes. It was revealed that IRF-1 also interacts with MyD88, and IRF-1, but not IRF-7, and is activated in response to TLR9 stimulation of cDCs to control the expression of IFN-β [76].

There are several models explaining the potency of pDCs in producing type I IFNs. One observation is that pDCs, but not other cell types, constitutively express IRF-7 [77]. Alternatively, another report demonstrated that retention of CpG-DNA in the endosomes of pDCs may facilitate interaction between CpG-DNA and the TLR9 complex [78,79]. It has been shown that A/D-type ODN is localized at the endosomes for a long time in pDC. In contrast, B/K-type ODN was localized at lysosomes in pDCs, and was processed more quickly than A/D-type ODN. Consequently, A/D-type ODN but not B/K-type ODN induces IFN-α production whereas B/K-type ODN induced maturation of pDC, and both types of ODN induce cytokine production in pDC. In both cases, TLR9 complexed with MyD88, IRAK-1, and IRF-7 resided at the CpG-ODN containing endosome to achieve robust TLR9 signaling. However, it was known that low concentrations of B/K-type CpG-ODN induced production of IFN-α in pDCs, implicating that another mechanism may be responsible for the difference in the action between A/D-type and B/K-type CpG-DNA [21]. However, the reason why pDCs, but not cDCs produce high amounts of IFN-a is not clearly understood.

To identify and monitor type I IFN producing cells *in vivo*, we generated a reporter mouse strain which expresses green fluorescence protein under the control of the *Ifna6* promoter [56]. Intravenous inoculation of CpG-ODN to *Ifna6*^{gfp} knockin reporter mice led to the expression of GFP exclusively in pDCs, but not in cDCs, IKDC or macrophages in the spleen. This is consistent with former reports that showed pDC is a main IFN-α producer in response to MCMV infection [15]. This data indicates that type I IFN producing cells which respond to TLR9 ligands are exclusively pDCs (Fig. 3). However, the importance of TLR9 in virus-induced type I IFN production is not fully understood yet. Although Herpes simplex virus infection highly induces production of IFN-α in pDCs, other cell types also produce type I IFNs independent of TLR9 [80,81]. Our studies with Newcastle disease virus (NDV), a RNA virus recognized by TLRs in pDCs, in *Ifna6*^{gfp} mice revealed that not only pDCs, but also cDCs and macrophages, are potent type I IFN producers to systemic infection [56]. However, cDCs and macrophages relied on the RLH system, but not on the TLRs. Furthermore, lung infection with NDV did not activate production of IFN-α in pDCs, and alveolar macrophages and cDCs are the main source of IFNs. These results argue the previous emphasis on the role of pDCs in RNA virus-induced type I IFN production. Although extensive studies of type I IFN producing cells in DNA virus infection has not yet been done, the importance of TLR9-mediated DNA virus recognition in pDCs will hopefully be elucidated using *Ifna6*^{gfp} mice in the near future.

6. TLR9 in autoimmunity

Type I IFN is known to be important for the pathogenesis of certain autoimmune diseases like systemic lupus erythematosus

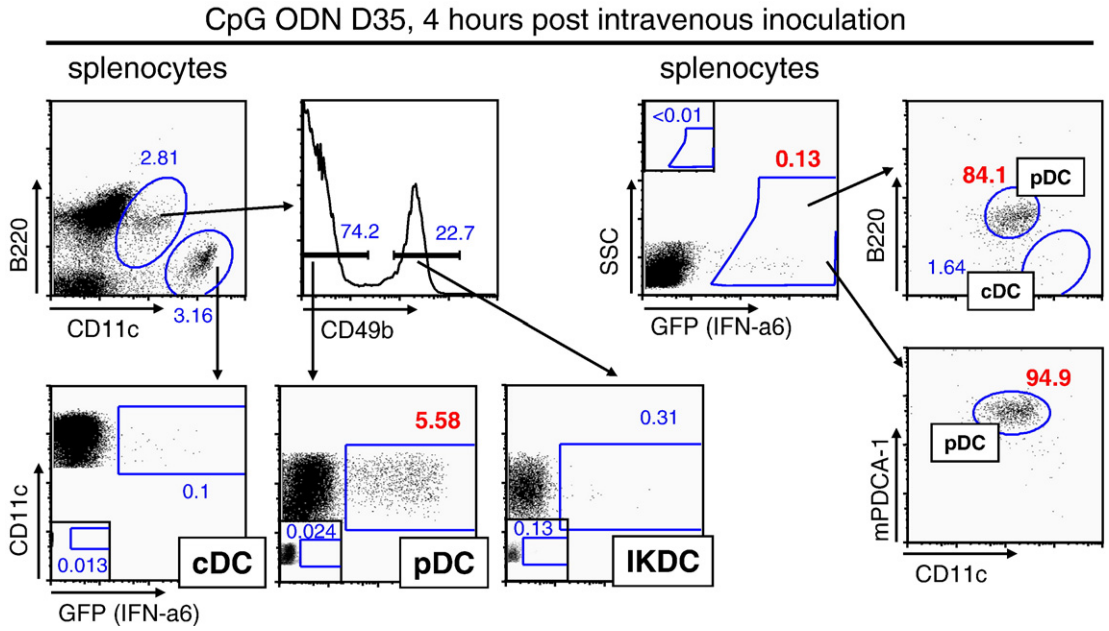

Fig. 3. Monitoring production of IFN-α from pDC upon TLR9 ligand stimulation *in vivo*. Knockin mice in which GFP is expressed under the control of the *Ifna6* gene promoter were intravenously inoculated with A/D-type CpG ODN D35 or PBS (inset). At 4 h after inoculation, splenocytes were prepared and analyzed using FACS. Live cells were gated and further fractionated into cDC ($CD11c^{hi}$), pDC ($CD11c^{dull}B220^{+}CD49b^{-}$), and IKDC ($CD11c^{dull}B220^{+}CD49b^{+}$). Cells of these fractions were analyzed for their GFP expression. In turn, GFP positive cells were analyzed for their expression of surface molecules. The data shown is partly adapted from [56] with some additional data and modifications.

(SLE) (reviewed in [82]). IFN-α levels in sera from SLE patients are known to be correlated with the severity of the disease. Moreover, SLE was found to be induced during the course of type I IFN therapy. From these observations, some researchers hypothesize that excess levels of type I IFN breaks peripheral tolerance and consequently leads to autoimmunity.

In SLE patients, anti-nuclear antibodies (ANA) are frequently detected. This is an autoantibody for chromatin and ribonucleoprotein (RNP) and is one of the hallmarks of SLE. Chromatin components complexed with autoantibodies can be ligands for TLRs [83–85]. Given that TLR9 is important for the production of type I IFNs in pDCs, TLR9 signaling is recently implicated in the pathogenesis of autoimmunity, especially in SLE.

Some patients with autoimmune disorders including SLE often have mutations in genes coding DNA nucleases, such as DNase-I [86], DNase-II [87], and DNase-III [88]. These mutations may affect the clearance of unnecessary DNA from dead cells and consequently the undigested self DNA cannot be controlled to expose to immune cells. The ANA–DNA complex is internalized by endosomes in pDC through Fcγ receptors [89,90]. A recent report indicates that pDC introduces self DNA coupled with antimicrobial peptide LL37 (also know as CAMP) into the endosome in an acidification-dependent manner [91]. In B cells, self DNA is captured by IgMs specific for self DNA [83]. The captured DNA-IgM complex is internalized and is retained longer in the cells. Then the DNA is recognized by TLR9 in B cells and the cells are activated. Once DNA is internalized into the endosomal compartment, TLR9 can access it and elicit signaling leading to an immune response. This is a first step for TLR9 to facilitate autoimmunity.

pDCs also produce type I IFN if they are activated by self DNA complexed with ANA or LL37 [91,92]. The production of type I IFNs, which may induce a strong adjuvant effect, is quite detrimental for autoimmunity. In B cells, TLR9 stimulation leads to class switching in TLR9 and a MyD88 dependent manner. Then the self reacting B cells undergo production of IgG2a and IgG2b specific for self DNA, also worsening the pathology of autoimmunity. Accordingly, autoimmune diseases advance in a "positive" feedback loop through TLR9 signaling and consequent activation of the immune system. Indeed, exogenous TLR9 ligands exacerbate the autoimmune response. However, the trigger of this feedback loop remains unclear, although a recently identified LL37-mediated mechanism will provide some answers for this problem.

Despite these findings, in some mouse models of autoimmunity, deficiency in TLR9 often shows a curing effect on pathology. In the common mouse autoimmunity model, the *lpr/lpr* mouse, which has a mutation in the *Fas* gene, anti-self RNP antibody is produced as well as ANA [93]. The RNP complex (containing the anti-RNP antibody) is reported to be a TLR7 ligand, and may act on TLR7 through RNAs contained in the complex [84,85]. On the other hand, TLR9-deficiency has been shown to exacerbate the pathology and increases the mortality of these mice together with the further increase in anti-RNP antibody titer[94]. The exacerbation of autoimmune diseases in TLR9-deficient species was observed in another autoimmune mouse model which had a mutant phospholipase Cγ2 [95], indicating that the role of TLR9 is a more general one in the pathology of autoimmune diseases.

Overall, TLR9 has a pivotal but paradoxical role in the pathology of autoimmunity. TLR9 can suppress the pathology

of autoimmunity in certain cases, although it may also act as a trigger and a center for a feedback loop of autoimmunity. The reason why such paradoxical effects occur is currently unknown.

7. Concluding remarks

A vast array of data indicates that TLR9 plays a key role in DNA-induced innate immunity, and links it with a role in acquired immunity through the activation of various cell types, such as pDC, cDC, and B cells. Its intracellular signaling pathway has been elucidated at the molecular level. The ligands for TLR9 have also been extensively studied. Several biological studies have concluded that spatial and temporal regulation is critical for proper and optimal TLR9 signaling. As well, the various cell types utilizing TLR9 have been defined. In this review, the mechanism of TLR9 at both the molecular and cellular levels have been discussed. Moreover, signaling through TLR9 has been shown to possibly contribute to some autoimmune diseases.

Despite these elucidations, there are still problems in translating this molecular and cellular level knowledge into the *in vivo* outcome of the immune responses. Of note, accumulating evidence has indicated that double-stranded DNA is recognized in the cytoplasm in a TLR-independent manner and can induce I IFN production and an antiviral response [19]. Undigested DNA from apoptotic cells also has this activity [96]. Excess excitation of this cytosolic pathway as well as the TLR9 pathway seems to lead to autoimmunity [97]. Furthermore, DNA from intracellular growing bacteria, such as Listeria, was also reported to stimulate host immune cells in a TLR9-independent manner [98]. Recently, a potential cytoplasmic DNA receptor named DAI (also known as DLM1 or ZBP1) was identified [99]. Further elucidation of the molecular mechanism of this pathway will greatly promote the understanding of DNA-mediated immune responses.

In addition to understanding these molecular mechanisms, precise knowledge about cell types, timing, and the location where TLR9 signaling takes place will reveal the true *in vivo* aspects of immunity evoked by TLR9. To answer such questions, imaging methods and the accompanying establishment of reporter systems *in vivo* will be quite useful. Several reports indicate that the structure of lymphatic tissue is important in order to mount an optimal immune response [100]. *In vivo* imaging experiments have also highlighted the importance of timing and location for immunity [101].

Preclinical studies for therapeutic use of DNA are also being performed not only in rodents, but also in non-human primates and humans. CpG-ODN is well known as an adjuvant for conventional vaccines. Administration of CpG-ODN with Fms-like tyrosine kinase 3-ligand (Flt3L) to rhesus has shown to induce strong vaccination activity against simian immunodeficiency virus in combination with plasmid DNA coding components of the virus [102]. CpG-ODN itself can induce strong Th1 immune response and consequently it shows protective activity against bacterial infection [103]. Moreover, CpG-ODN exhibits anti-tumor activity with increasing T cell response and NK cell response as mentioned above [104]. Based on these results, several DNA vaccines have been already begun clinical trials [105].

In spite of these results, this review has emphasized the basic research available on TLR9 signaling and its related immune phenomena. However, we believe that future research on the mechanism of TLR9-mediated immunity, at both the molecular and the individual level, will help us better understand the mode of action of DNA vaccines and the pathology of autoimmunity.

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