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Regulation of Nucleic Acid Sensing Toll-Like Receptors in Systemic Lupus Erythematosus

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1. Introduction

Autoimmune disease is an aberrant response of the immune system to self. Unlike the adaptive immune system, the innate immune system is not selected during development and was originally thought to inherently discriminate between host and foreign molecular structures (Janeway, 1989). This is true for many innate immune receptor ligands including lipopolysaccharide, which is only synthesized by Gram-negative bacteria. However, some innate immune receptors detect nucleic acids that are shared between microbes and the host. Immune complexes containing nucleic acids are a hallmark of Systemic Lupus Erythematosus (SLE), and the nucleic acid sensing Toll-like receptors (TLRs) respond to the DNA and RNA within these complexes thereby contributing to disease. Defects in regulation of this class of innate immune receptors likely play a key role in precipitation of disease. Here we review nucleic acid sensing TLRs in SLE and recent advances in our understanding of the regulatory mechanisms governing TLR activity. Since breakdown of regulatory mechanisms controlling response of nucleic acid-sensing TLRs likely contributes to development of SLE, targeting specific proteins in these regulatory pathways has the potential to block nucleic acid-driven autoimmune inflammation.

2. Nucleic acid sensing toll-like receptors in SLE

2.1 Toll-like receptors

Toll-like receptors (TLRs) are a family of innate immune receptors that directly detect molecular structures and initiate signaling. Engagement of TLRs initiates innate immune responses that promote microbial killing and antigen presentation to educate T cells and B cells. At least 10 different TLRs recognize various microbial structures such as lipopeptides (TLR1, TLR2, and TLR6), lipopolysaccharide (TLR4), bacterial flagellin (TLR5), double stranded RNA (dsRNA, TLR3), single stranded RNA (ssRNA, TLR7, TLR8), and single stranded DNA (ssDNA, TLR9) (Takeda, et al. 2003).

TLRs are type 1 transmembrane receptors with C-termini facing the cytoplasm of the cell and ectodomains either at the cell surface or in the lumen of intracellular compartments. The cytoplasmic domain has homology with the IL-1 and IL-18 receptors and has been called the Toll/IL-1-like receptor domain (TIR). The three-dimensional structures of two TLR cytoplasmic TIR domains have been solved and are globular with many surfaces for protein-protein interactions (Xu, et al. 2000). The TIR domain associates with several adapter

proteins that initiate signal transduction. These adapters include myeloid differentiation factor 88 (MyD88), and TIR-domain-containing adapter-inducing interferon- β (TRIF, also known as MyD88 adapter-like, MAL), which promote production of proinflammatory cytokines and type I interferons. The ectodomain is composed of a series of leucine rich repeats that form a curved solenoid. Alignment studies predicted a model structure for the ectodomains of TLRs (Bell, et al. 2003), which was supported by crystallographic studies (Bell, et al. 2005; Choe, et al. 2005). TLRs are expressed on a wide variety of cell types including B cells, T cells, dendritic cells, macrophages, and intestinal epithelial cells, although different cell types have unique repertoires of TLR expression (Takeda, et al. 2003). For example, human plasmacytoid dendritic cells express the nucleic acid-sensing TLRs, TLR7 and TLR9. TLR7- and TLR9-dependent responses by both B cells and plasmacytoid dendritic cells have been proposed to contribute to SLE (Marshak-Rothstein, 2006).

2.2 Nucleic acids as TLR ligands

Multiple nucleic acids, including ssRNA, dsRNA, and DNA, induce inflammatory responses. For DNA, the response is dependent on a 5'-cytosine-guanosine-3' dinucleotide (CpG). The central cytosine must be unmethylated, and is active when surrounded by specific bases, which together form the CpG motif (Krieg, 2002). These CpG motifs are rare in vertebrate DNA due to reduced frequency of the CG dinucleotide (CpG suppression) and increased frequency of cytosine methylation (Cardon, et al. 1994; Klinman, et al. 1996; Krieg, et al. 1995). However, CpG motifs are present and functional in bacterial DNA, in plasmid DNA produced in bacteria, and in synthetic DNA. Variation of sequence and physical structure of synthetic DNAs have resulted in characterization of at least four types of CpG oligodeoxynucleotides each with different activity on cells. Three are stimulatory, and one is inhibitory (Gursel, et al. 2003; Verthelyi, et al. 2001). Inhibitory DNAs do not require a CpG motif and the mechanism of inhibition has not been clearly defined (Gursel, et al. 2003; Krieg, et al. 1998; Lenert, et al. 2003). Type A CpG DNAs (also called D) induce robust type I interferon production, while type B CpG DNAs (also called K) induce B cell proliferation and proinflammatory cytokine production (Verthelyi, et al. 2001). The last class type C CpG DNAs have properties of both type A and type B CpG DNAs and thus induces both types of cellular responses (Vollmer, et al. 2004).

Regardless of their class, CpG DNAs require endocytosis and acidification of endosomes for activity (Figure 1). Blockade of uptake by immobilization of the CpG DNA on beads inhibits the B cell proliferative activity of synthetic CpG DNAs (Manzel & Macfarlane, 1999). Inhibition of endosomal acidification blocks CpG DNA-induced cytokine release by macrophages (Hacker, et al. 1998). Furthermore, cellular activation by CpG DNA initiates on endosomes (Ahmad-Nejad, et al. 2002). Vertebrate DNAs are poorly internalized, which contributes to their poor stimulatory activity. However, vertebrate DNA is stimulatory when in complex with proteins such as high mobility group box 1 (HMGB1), the antimicrobial peptide LL37, or anti-DNA antibodies (Lande, et al. 2007; Leadbetter, et al. 2002; Tian, et al. 2007). Whether the CpG DNA induces proinflammatory cytokines or type I interferon also depends on the endosomal compartment where the DNA is retained (Honda, et al. 2005). Honda and colleagues demonstrated that different types of DNA were trafficked to and retained within different endosomal compartments. For example, the type I interferon inducing CpG DNAs (type A) rapidly co-localized with FITC-dextran, a marker for early endosomes, but failed to co-localize with lysosomal markers. In contrast, rapid

localization with lysosomal markers correlated with proinflammatory cytokine production induced by type B CpG DNAs. In another study the outcome of cellular responses to the DNA types could be swapped by changing the physical and chemical properties of the DNA (Guiducci, et al. 2006). Multimerization of type B CpG DNAs, so that their physical structure resembled type A CpG DNAs, caused them to be retained in early endosomes and induce A-type responses. Response of B cells was also dependent on CpG DNA type and delivery mechanism (Avalos, et al. 2009). These studies strongly correlated location of DNA detection with cellular outcome and suggested that manipulation of localization and receptor recognition could change the outcome of cellular response.

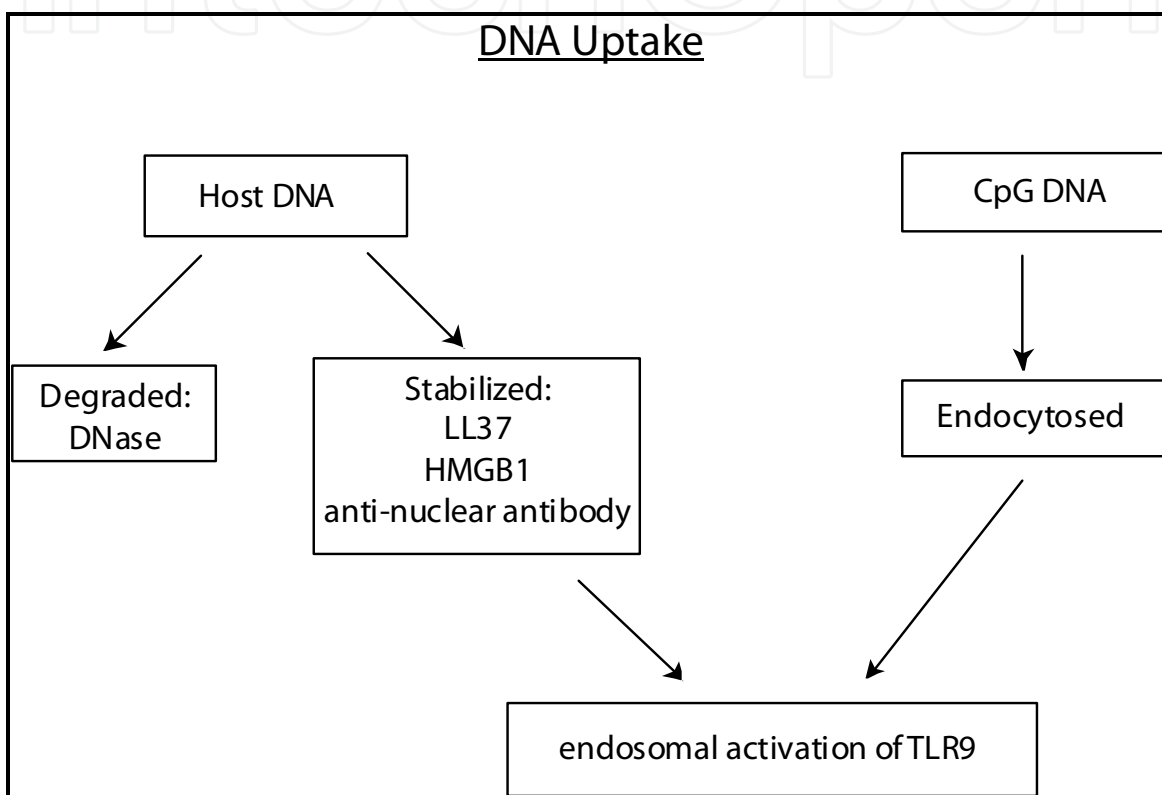


Fig. 1. DNA uptake into the cell. Host DNA is degraded in the extracellular space by DNase and does not normally induce inflammatory responses. However, several host proteins including LL37, HMGB1, and anti-nuclear antibodies stabilize host DNA and facilitate uptake into the endosomal compartment. Endocytosed host DNA activates TLR9. Unlike host DNA, synthetic CpG DNAs, or bacterial DNA, are efficiently endocytosed and activate TLR9.

At concentrations of CpG DNA used by many investigators, uptake occurs by fluid phase endocytosis. Uptake of CpG DNA reached a plateau at 2 hours, was slowed at low temperature and was inhibited by known endocytosis inhibitors such as sodium azide and cytochalasin B (Yakubov, et al. 1989). Yet, at lower concentrations, uptake was significantly more efficient, which indicated that the CpG DNA was internalized by receptor-mediated endocytosis. Using radiolabeled cells, multiple groups demonstrated specific DNA binding proteins that likely assisted in internalization and trafficking of CpG DNA to the correct endosomal compartment where it encountered TLR9 (Beltinger, et al. 1995; Yakubov, et al. 1989). However, the identity of these receptors remains unknown. During the

internalization process, CpG DNA transits through both early and late endosomes, which is important because, as mentioned above, CpG DNAs trigger different cellular outcomes depending on the compartment where signaling initiates.

A hallmark of SLE is elevated serum antibodies to nuclear components, which enhance response to DNA (Leadbetter, et al. 2002). Immune complexes from SLE patient serum were enriched in CG content within the DNA, consistent with increased presence of potential stimulatory motifs (Sano & Morimoto, 1982). Complexes with DNA bound to anti-idiotypic B cell receptors (rheumatoid factor) and to Fc receptors to mediate internalization (Boule, et al. 2004; Leadbetter, et al. 2002). By either mechanism the uptake was very efficient and delivered the complexes to the endosomal compartment. Synergistic cytokine production and autoantibody induction by enhanced uptake of DNA-containing immune complexes likely contributes to the induction and propagation of anti-nucleic acid antibody production so frequently observed in SLE.

Internalization of CpG DNA and host DNA is also facilitated by several other host proteins including high mobility group protein 1 (HMGB-1), and the antimicrobial peptide LL37. The cationic antimicrobial peptide LL37 was highly elevated in the skin of patients with psoriasis (Lande, et al. 2007). LL37 formed a stable complex with host DNA, and induced a TLR9-dependent, DNase-sensitive, type I interferon response from human plasmacytoid dendritic cells. The nuclear factor HMGB1 is retained within cells under normal conditions, but cell death and inflammation releases HMGB1, which formed a stable complex with host DNA. Association of CpG DNA-A with HMGB1 dramatically enhanced production of type I interferon (Tian, et al. 2007). In fact, HMGB1 was found in immune complexes with host DNA. Altogether, these studies demonstrate that while host DNA alone is not very immunostimulatory, association with a variety of different proteins, present in disease states, promote endosomal uptake where the DNA can associate with TLRs and induce pathologic interferon responses.

2.3 The role of TLRs in B cells and DC in SLE models

Mouse models have provided significant experimental evidence for the importance of TLRs in SLE. Deficiency in UNC93B1, a chaperone-like protein required for endocytic trafficking of nucleic acid-sensing TLRs, results in less severe disease in induced models of SLE in mice (Kono, et al. 2009). Furthermore, UNC93B1 protein is upregulated in SLE (Nakano, et al. 2010), underscoring the importance of TLRs in disease manifestations. The Y-linked autoimmune accelerator (Yaa) mutation, which accelerates and enhances spontaneous disease on the B6/FcRIIB $-/-$ background, is due to TLR7 gene duplication (Pisitkun, et al. 2006), and disease in Fas-deficient MRL/lpr mice is dependent on TLR7 expression (Christensen, et al. 2006). Together these studies show that nucleic acid-sensing TLRs contribute to SLE.

The role for TLR9 in SLE-like disease in mice is less clear. TLR9 deficient mice have reduced anti-DNA antibodies, but enhanced disease (Christensen, et al. 2005). Despite these limitations, much is known about the regulation of TLR9, and TLR9 remains a good model to study the regulation of nucleic acid-sensing TLRs. A specific role for TLR9 in B cell responses to DNA containing immune complexes was supported by studies using a mouse expressing a transgenic "rheumatoid factor" receptor. In vitro stimulation with immune complexes that included DNA, such as anti-nucleosome complexes, induced proliferation of the transgenic B cells, but non-nucleic acid complexes, such as BSA-anti-BSA complexes did not. The response was DNase sensitive and dependent on MyD88 and uptake of the

immune complexes into the endosomal compartment (Leadbetter, et al. 2002). Therefore, while TLR9 may not be a major contributor to disease in patients, defects in TLR9 regulation may have very specific functional consequences and a better understanding of the regulatory mechanisms may provide necessary information to therapeutically target TLR9 and other nucleic acid-sensing TLRs.

Patients with SLE have high levels of type I interferons in the blood and their peripheral blood mononuclear cells have a characteristic upregulation of interferon regulated genes (Bennett, et al. 2003). Plasmacytoid dendritic cells circulating in the blood are a major source of type I interferons and were originally called natural interferon producing cells (NIPCs). In humans, this subset of dendritic cells expresses predominantly TLR7 and TLR9 (Hornung, et al. 2002; Krug, et al. 2004). Production of type I interferons by plasmacytoid dendritic cells can be induced by immune complexes produced in abundance in patients with SLE (Ronnlom & Alm, 2001). Dendritic cells have Fc receptors on their cell surface that capture and internalize immune complexes, which induces interferon in a CpG motif- and TLR9-dependent manner (Boule, et al. 2004; Yasuda, et al. 2009). High circulating type I interferon correlates with immune pathology and disease severity in SLE. Therefore, TLR9 contributes to interferon production, and interferon can in turn augment TLR9-dependent responses.

2.4 Nucleic acid-sensing TLRs detection of self-ligands

When the stimulatory activity of CpG DNA was first described, it was thought to represent microbial DNA and that self-DNA was non-stimulatory. This was due to the requirement for the central CG dinucleotide, unmethylation of the C, and for selectivity for surrounding bases. These arguments held for many years, but it was known, even then, that the mammalian genome had the potential to induce TLR9-mediated responses (Ishii, et al. 2001; Yasuda, et al. 2005). Many studies, including the ones reviewed below, have been focussed on understanding what regulates TLR9 mediated responses and why self-DNA is not normally detected. However, recent studies suggest that TLR9 signaling is important for normal responses like wound repair.

Cells go to great lengths to assure DNA is not released into the extracellular milieu when they die by condensing and digesting their DNA during apoptosis. However, necrosis and neutrophil extracellular trap (NET) formation intentionally releases DNA that can induce inflammatory responses (Garcia-Romo, et al. 2011; Lande, et al. 2011; Villanueva, et al. 2011). Interestingly, SLE neutrophils were primed by high type I interferon levels in vivo, and in response to immune complexes, the neutrophils from SLE patients generated NETs that had a high content of DNA, LL37 and HMGB1. These proteins protected the DNA from degradation and facilitated internalization, and thereby increased the inflammatory potential of the host DNA. Therefore, since it is purposefully released under certain conditions, there must be other regulatory mechanisms to avoid response to host DNA. DNase is present in serum and in the extracellular environment and degrades potentially stimulatory host DNA. DNase deficient mice were born healthy but develop lupus like disease around six months of age (Napirei, et al. 2000). Heterozygous mice had increased serum concentrations of anti-nuclear antibodies, and more glomerulonephritis. However, in homozygous DNase deficient mice these SLE parameters were even higher. Mutations in DNase have been identified in SLE patients (Yasutomo, et al. 2001), and together these studies suggest that DNase is an important mechanism to prevent response to host DNA.

Interestingly, recent studies have shown that detection of self-DNA may be a normal biological process, and is, in fact, critical for wound healing (Gregorio, et al. 2010; Sato, et al. 2010). In the absence of TLR9, full thickness biopsy wound healing was delayed, and application of CpG DNA enhanced healing in a TLR9 dependent manner (Sato, et al. 2010). These data suggest that TLR9 plays an important role in wound healing. In a different model, tape stripping-induced epidermal injury induced plasmacytoid dendritic cell and neutrophil infiltration (Guiducci, et al. 2010). This response was accompanied by production of type I interferon, and was dependent on the signaling adapter molecule MyD88. Treatment of wild-type mice with a TLR7-TLR9 inhibitor inhibited the response, implicating these TLRs in the process. In lupus-prone mice, the same tape-stripping procedure led to chronic wounds with a type I interferon signature that resembled SLE skin lesions. Therefore, detection of DNA and RNA by TLR9 and TLR7 is important for normal wound healing. Dysregulation of this pathway in SLE likely contributes to autoimmune inflammation, especially in the skin, and is a potential target for therapeutic intervention.

3. Regulation of TLR9

3.1 Compartmentalization of TLR9

Infectious agents replicate in various locations outside and inside cells. Bacteria and viruses are internalized into endosomes, and some can escape into the cytoplasm. Therefore, positioning of TLRs is important for detecting components of microbes in the varied locations where they can reside. Some TLRs, such as TLR2, TLR4, and TLR5, are expressed at the cell surface to detect ligands expressed on the surface of bacteria (lipopolysaccharide, TLR4; lipopeptides, TLR2; and flagellin, TLR5). However, nucleic acids, such as DNA and RNA, are encapsulated within bacteria and viruses, and are only released upon internalization into endosomes. To accommodate this, nucleic acid sensing TLRs are localized intracellularly. For example, TLR9 is primarily found in the endoplasmic reticulum (ER) of resting cells (Latz, et al. 2004; Leifer, et al. 2004) where it colocalizes with ER, and not endosomal, markers. Since detection of DNA occurs in endosomes, these data suggest that there is an induced trafficking event that leads to TLR9 entry into this compartment (Leifer, et al. 2004). The unique compartmentalization of nucleic acid-sensing TLRs has been proposed as a major regulatory mechanism to prevent response to host DNA (Barton, et al. 2006). Fusion of the ectodomain of TLR9 with the transmembrane domain and cytoplasmic tail of TLR4 created a protein that localized to the cell surface. This change in localization endowed the TLR9 ectodomain with the ability to respond to host DNA (Barton, et al. 2006). These data support a model where TLR9 is specifically trafficked intracellularly to avoid access to the extracellular milieu, thereby preventing recognition of host DNA.

3.2 Trafficking of TLR9 through the Golgi to localize in the endolysosomes

While TLR9 predominantly resides in the ER it must traffic to the endosomal compartment where it encounters endocytosed CpG DNA (Latz, et al. 2004; Leifer, et al. 2004) (Figure 2). Normally, transmembrane or secreted proteins synthesized in the ER traffic through the Golgi to access the cell surface or intracellular endosomes. However, TLR9 was sensitive to endoglycosidase H (endo H) treatment, which indicated that TLR9 had not reached the

Golgi (Latz, et al. 2004). In 2009 Chockalingam et al., showed that Brefeldin A inhibited TLR9 response to CpG DNA (Chockalingam, et al. 2009). Since Brefeldin A is a small molecule that inhibits transport of proteins from ER to Golgi, TLR9 signaling appeared to be dependent on Golgi trafficking.

When proteins traffic through the Golgi, the high mannose glycans are processed to hybrid forms that are still cleaved by Endo H; therefore, highly specific lectins were used to determine whether TLR9 had glycan modifications indicative of Golgi transit (Chockalingam, et al. 2009). Lectins are plant proteins that selectively recognize carbohydrate structures. For example, *Datura stramonium* (DS) lectin specifically recognizes "Gal β 1 \rightarrow 4GlcNac" structures present only on proteins that have been processed by Golgi resident enzymes. DS lectin bound to TLR9, which confirmed that TLR9 trafficked through the Golgi during synthesis (Chockalingam, et al. 2009). TLR9 immunoprecipitated from the lysosomal compartment of HEK293 cells also bound DS lectin, and co-immunoprecipitated with the signaling adapter MyD88 (Chockalingam, et al. 2009). These data indicated that lysosomal TLR9 had transited through the Golgi and contributed to signaling.

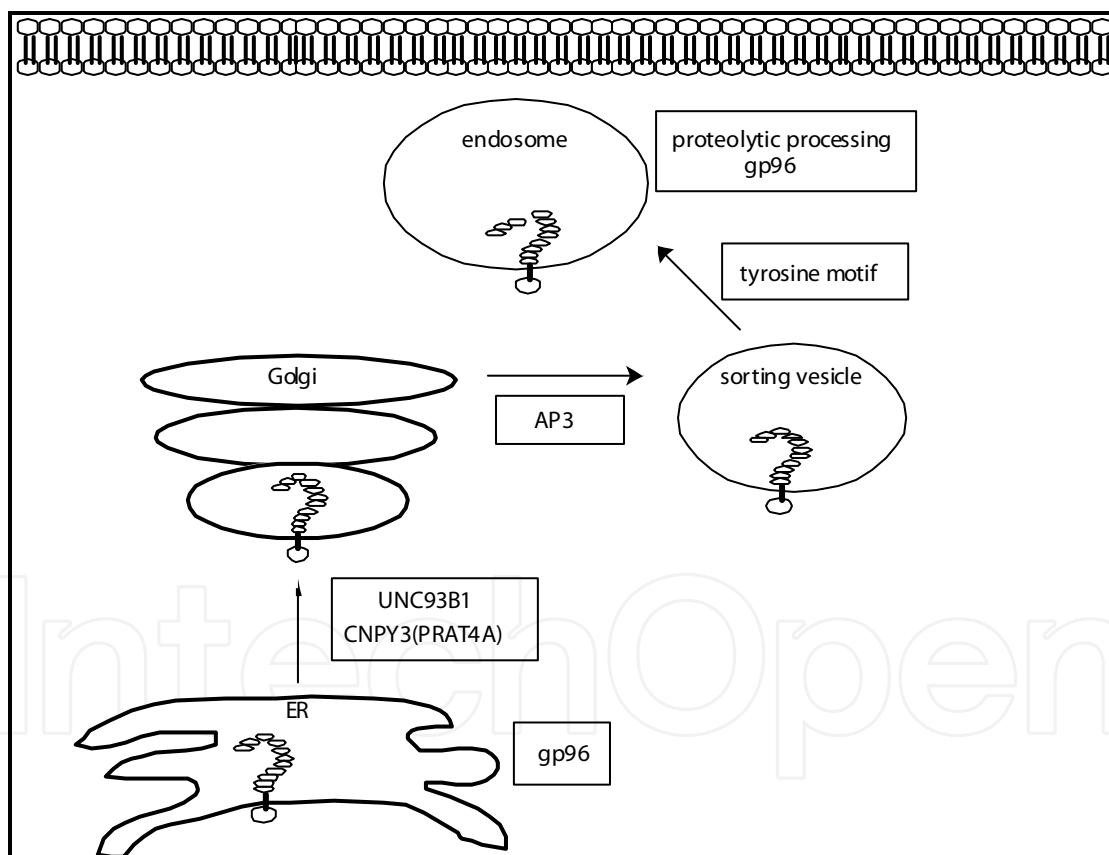


Fig. 2. Regulation of TLR9 trafficking. After synthesis in the ER TLR9 associates with gp96. TLR9 traffics out of the ER to the Golgi in a manner dependent on gp96, PRAT4A, and UNC93B1. TLR9 traffics from the Golgi to a sorting vesicle in an AP3 dependent manner where it is then sorted to the endosomal compartment via a cytoplasmic tyrosine motif. In the endosomal compartment TLR9 is proteolytically processed either active or negative regulatory forms that modulate TLR9 signaling. Lack of gp96 prevents TLR9 proteolytic processing.

3.3 Proteins that regulate intracellular localization and trafficking

Several proteins are critical for TLR9 trafficking both out of the ER and to the endosomal compartment (Figure 2): UNC93B1, adapter protein 3 (AP3), a protein associated with TLR4 (PRAT4A), Slc15a4, and glycoprotein 96 (gp96, also known as glucose regulated protein 94 (gp94). Recessive N-ethyl-N-nitrosourea-induced mutagenesis revealed a mouse line that lacked response to TLR3, TLR7, and TLR9 ligands. These mice had a single point mutation (H412R) in UNC93B1 (Tabeta, et al. 2006). In dendritic cells from these mice, TLR7 and TLR9 did not localize to the endosomal compartment (Kim, et al. 2008). Interestingly, UNC93B1 seems to play opposing roles in regulation of TLR7 and TLR9 (Fukui, et al. 2009). Reconstitution of UNC93B1 deficient cells with UNC93B1 containing a single point mutation (D34A) resulted in hyperresponsiveness to TLR7, yet hyporesponsiveness to TLR9, ligands. Therefore, the role of UNC93B1 in regulation of nucleic acid sensing TLRs is clearly important and interfering with UNC93B1 function has different effects on signaling by different TLRs.

Two ER luminal proteins, gp96 and PRAT4A, are essential for TLR9 exit from the ER. PRAT4A, also known as CNPY3, bound to TLR9, which depended on methionine 145 of PRAT4A (Kiyokawa, et al. 2008). In the absence of PRAT4A, TLR9 did not access endosomes, and PRAT4A deficient cells lacked response through all TLRs, except TLR3 (Takahashi, et al. 2007). The heat shock protein gp96 also bound to TLR9 and was required for B cell and macrophage response to CpG DNA (Randow & Seed, 2001; Yang, et al. 2007). A pre-B cell line with a frame-shift mutation in gp96 was 10,000 times less sensitive to LPS than the non-mutant line, which was due to a lack of TLR4 on the cell surface (Randow & Seed, 2001). This study suggested that gp96 regulated trafficking of TLRs. Further studies using a mouse with macrophage specific knockout of gp96 showed that gp96 is essential for TLR9 trafficking and signaling, and was in fact a chaperone for all TLRs except TLR3 (Liu, et al. 2010; Yang, et al. 2007). In 2011 Liu et al., demonstrated that gp96 and PRAT4A directly interacted to form a multimeric complex with TLR9 (Liu, et al. 2010). Very recent studies using gp96 specific inhibitors have examined the role of gp96 after TLR9 synthesis and trafficking is complete. These studies show that gp96 remains associated with TLR9 in the endosomal compartment and that specific inhibitors block CpG DNA signaling and cause a loss of TLR9 protein (JC Brooks and CA Leifer unpublished observations). This suggests that gp96 has an additional function in regulating the conformational stability of TLR9 in the endosomal compartment.

Cytoplasmic proteins are also important for TLR9 trafficking. Plasmacytoid DCs from adapter protein 3 (AP3) deficient mice failed to induce a type I interferon response after CpG DNA stimulation despite normal IL-12 production (Sasai, et al. 2010). AP3 is a cytosolic protein that associates with endosomes and sorts transmembrane proteins from the endosomal compartment to lysosome-related organelles (Bonifacino & Traub, 2003). In the absence of AP3, TLR9 did not colocalize with markers for lysosome-related organelles (Sasai, et al. 2010). This group suggested that it was these lysosome-related organelles that were critical for induction of type I interferons (Sasai, et al. 2010). However, this conclusion contradicts previously published studies showing that initiation of signaling that results in type I interferon production occurs on early endosomes (Guiducci, et al. 2006; Honda, et al. 2005). In a separate study using the same AP3 deficient mice, both proinflammatory and type I interferon production were lost. Therefore, AP3 is important for TLR9-induced cytokine production, but its exact role in TLR9 biology remains unclear.

Recent data have shown that TLR9 signaling also depends on Slc15a4, a twelve-spanning transmembrane oligopeptide transporter that localizes to the endolysosomal compartment (Blasius, et al. 2010; Yamashita, et al. 1997). Cells from Slc15a4 deficient mice lack response to nucleic acid sensing TLRs (Blasius, et al. 2010). Again, the specific role of Slc15a4 remains unknown, but may involve endolysosomal transport of TLR9 or a TLR9-associated protein required for TLR9 function.

3.4 Specific motifs in TLR9 that regulate localization and trafficking

Localization of TLRs is regulated by sequences in their transmembrane domains and cytoplasmic tails. Fusion of TLR4 to the transmembrane and cytoplasmic tail of various TLRs resulted in distinct localizations of the chimeric proteins (Nishiya & DeFranco, 2004). For example, TLR4 by itself localized to the cell surface, and fusion of TLR4's ectodomain with the transmembrane and cytoplasmic tail of TLR1, TLR2, TLR5, or TLR6 resulted in similar localization. In contrast, when TLR4 was fused to the transmembrane and cytoplasmic tail of any of the nucleic acid-sensing TLRs, the resulting chimeric receptor was not detected at the cell surface. Further studies using different approaches identified different motifs in TLR9 responsible for this localization (Barton, et al. 2006; Leifer, et al. 2006). TLR9's ectodomain fused to TLR4's transmembrane and cytoplasmic domains localized to the cell surface (Barton, et al. 2006). The chimera retained the ability to respond to CpG DNA, yet was resistant to endosomal acidification inhibitors. Interestingly, TLR9 associates with UNC93B1 via the transmembrane domain and this may explain, in part, the requirement for this association in TLR9 signaling.

The cytoplasmic tail of TLR9 also contains a specific localization motif (Leifer, et al. 2006). In this study, the ectodomain of the IL-2 receptor alpha chain, which normally localized to cell surface, was fused to the transmembrane and cytoplasmic tail of different TLRs (Leifer, et al. 2006). A fusion with the TLR4 transmembrane and cytoplasmic tail localized to the cell surface; however, a fusion with the same regions of TLR9 was not. Truncation analysis revealed that deletion of all but four amino acids of the cytoplasmic tail generated a protein that was robustly expressed at the cell surface, ruling out a contribution of the transmembrane domain to intracellular localization. It is unclear why these two studies showed opposite requirements for TLR9 transmembrane domain. Regardless, additional truncations and mapping identified a 14 amino acid motif that was important for TLR9 intracellular localization. Follow-up studies showed that mutation of a critical tyrosine (888) within this motif abolished proinflammatory cytokine production. Interestingly, this mutant maintained normal interferon responses suggesting that this motif was required for trafficking TLR9 to the compartment selectively required for induction of proinflammatory cytokines (A Chockalingam and CA Leifer unpublished observations). It remains to be determined if this motif is necessary for association with AP3 or other regulatory proteins.

3.5 Proteolytic regulation of TLR9

In addition to trafficking to specific endocytic compartments, several recent studies have demonstrated that TLR9 is proteolytically processed in endosomes and that this processing regulates TLR9 function (Chockalingam, et al. 2011; Ewald, et al. 2011; Ewald, et al. 2008; Park, et al. 2008; Sepulveda, et al. 2009). The ectodomain of TLR9 contains 25 leucine rich repeats. The first 14 and the second 15 leucine-rich repeats are interrupted by a region predicted to have very little secondary structure, often referred to as the hinge (Bell, et al.

2003). The first described proteolytic event was mapped to this hinge region through a mass spectrometric approach (Park, et al. 2008). The form of TLR9 generated encompasses one-half of the ectodomain and all of the transmembrane and cytoplasmic tail (Figure 3). This proteolytic event is inhibited by endosomal acidification inhibitors and by broad-spectrum cathepsin inhibitors (Ewald, et al. 2008; Park, et al. 2008). Additional studies with specific cathepsin inhibitors, and in cathepsin deficient mice, did not reveal a unique cathepsin responsible for the cleavage. An independent study, showed an additional proteolytic event (Sepulveda, et al. 2009). While this study did not reveal the precise location of the proteolysis, a specific enzyme, asparagine endopeptidase was shown to be important. A more recent study suggested that stepwise processing of TLR9 is required to attain fully functional proteolytically processed TLR9 (Ewald, et al. 2011). Interestingly, knockdown of either PRAT4A (CNPY3) or gp96 by shRNA targeting resulted in a loss of proteolytic processing of TLR9, and suggested that these chaperones were required for TLR9 to access endosomes (Liu, et al. 2010).

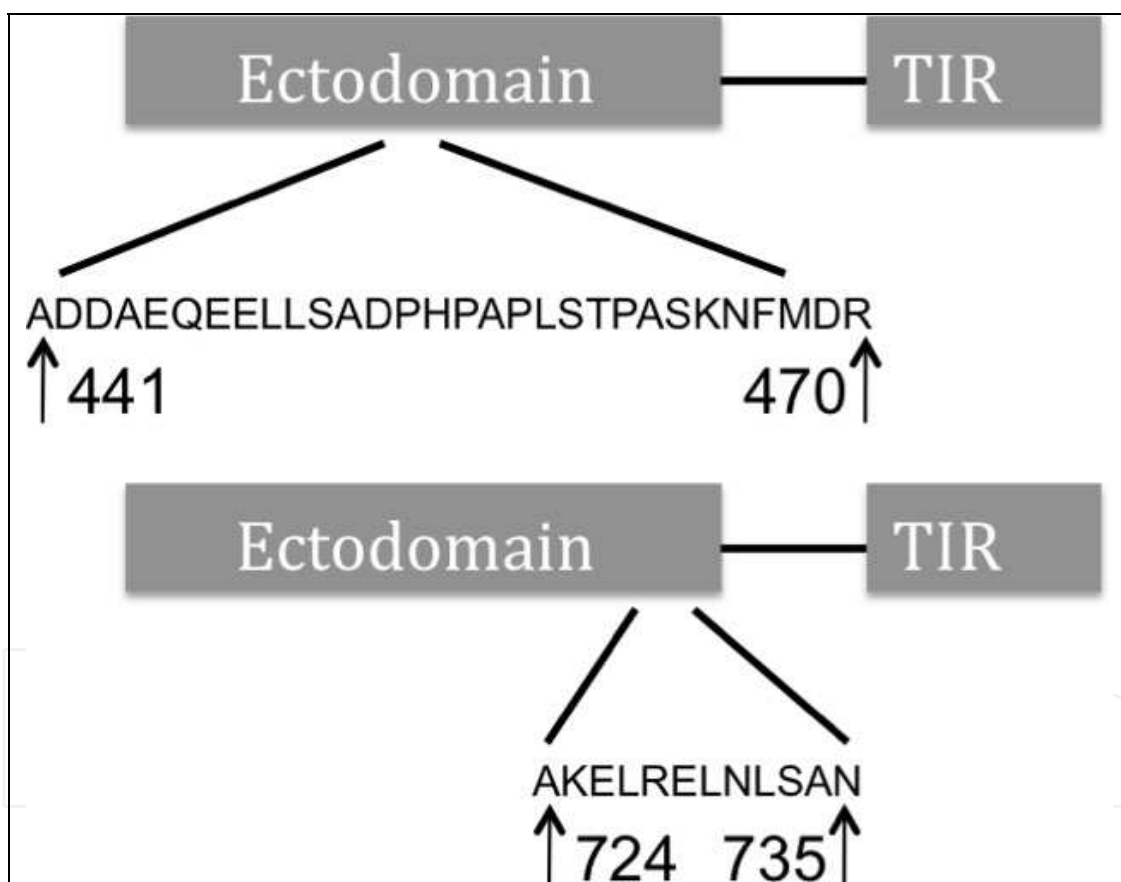


Fig. 3. Proteolytic processing of TLR9. The ectomain of TLR9 is proteolytically processed into two forms. The first form is active and consists of one half of the ectodomain of TLR9 and the transmembrane and cytoplasmic tail (amino acids 471-1032) The second form is a negative regulator of TLR9 signaling and consists of almost the entire ectodomain with no transmembrane or cytoplasmic tail. This form, called soluble TLR9, is released into endosomes, but is not secreted. By binding to internalized DNA, and to full-length TLR9, this form blocks signaling. The relative ratio of these two fragments likely determines the cellular response upon exposure to DNA.

TLR9 is also proteolytically processed at a completely different position to generate a negative regulator of TLR9 signaling (Chockalingam, et al. 2011). This proteolytic event resulted in generation of an intact ectodomain separated from the transmembrane domain and cytoplasmic tail (Figure 2). This soluble form of TLR9 bound to CpG DNA, associated with full length TLR9, and dominant negatively inhibited responses by the full-length receptor (Chockalingam, et al. 2011). In contrast to the other proteolytic cleavage events, generation of soluble TLR9 occurred in cells expressing endogenous TLR9 (Chockalingam, et al. 2011). Soluble TLR9 is likely important in regulating TLR9 responses since intestinal epithelial cells poorly responded to CpG DNA, and abundantly generated soluble TLR9 (Chockalingam, et al. 2011). Therefore, correlative studies on TLR9 in different pathological conditions must account for the complexity of TLR9 post-translational modification.

4. Conclusion

In this review we have highlighted recent studies describing regulatory mechanisms governing nucleic acid-sensing TLRs. While TLRs are critical for host defence against infection, some TLRs recognize ligands shared between infectious agents and the host (Marshak-Rothstein & Rifkin, 2007). Despite complex regulatory mechanisms, these TLRs do contribute to autoimmune disease (Marshak-Rothstein, 2006). Recent studies have revealed multiple post-translational mechanisms that regulate this family of TLRs, and if dysregulated could also contribute to the development of autoimmune disease. By exploiting mechanisms that control nucleic acid sensing TLRs we will relieve TLR-mediate autoimmune inflammation.

Host DNA and RNA are not typically inflammatory since there are several regulatory mechanisms to prevent availability of host DNA. These include preventing its release (apoptosis), and degrading it once it is released (DNase). Association with various host proteins such as HMGB1, LL37, and autoantibodies stabilize host DNA and enhance its uptake where it gains access to intracellular nucleic acid sensing TLRs (Lande, et al. 2007; Leadbetter, et al. 2002; Tian, et al. 2007). When this occurs, especially by anti-nuclear antibodies, host DNA and RNA enhance type I interferon production from dendritic cells and promote proliferation and antibody production from B cells. Defects in expression of the nucleic acid stabilizing proteins changes susceptibility to autoimmunity in mouse models. Development of drugs that block stabilization of DNA by these host proteins, and thereby block proinflammatory or interferon responses, will reduce autoinflammation and offer new ways to treat SLE.

The nucleic acid-sensing TLRs are also carefully regulated to avoid response to host DNA. To avoid detection of host DNAs and RNAs, nucleic acid-sensing TLRs are excluded from the cell surface (Latz, et al. 2004; Leifer, et al. 2004). Several proteins including UNC93B1, and gp96 regulate nucleic acid-sensing TLR access to endosomes (Kim, et al. 2008; Yang, et al. 2007). Specific parts of TLR9 have been found to be important for this regulation and may interact with some of the localization regulators (Barton, et al. 2006; Leifer, et al. 2006). Development of drugs that affect the activity of these proteins would change the intracellular distribution of TLRs, thereby affecting the ability of these receptors to detect and respond to nucleic acids.

Once nucleic acid-sensing TLRs do reach endosomes, they are proteolytically processed (Chockalingam, et al. 2011; Ewald, et al. 2008; Park, et al. 2008), which modifies their

function. Cleavage at two different locations within the ectodomain leads to either activation (Ewald, et al. 2008; Park, et al. 2008), or inhibition (Chockalingam, et al. 2011), of TLR9 activity. Specific identification of the enzymes responsible for these proteolytic events will provide new targets for drug development to interfere with TLR signaling and response to host DNA.

When host DNA, or RNA, does activate a TLR, it induces robust inflammation and production of pathologic levels of cytokines such as type I interferon. While several contributing factors to the development of SLE have been found, new data on post-translational regulation of nucleic acid-sensing TLRs show that we still have much to learn. Dysregulation in any of these regulatory proteins will change the intracellular localization of TLR9 and could potentially lead to aberrant response to host nucleic acids. Identifying these regulatory pathways is the first step to understanding how defects in these pathways lead to disease. Specifically targeting these regulatory proteins within these pathways will reduce, or restore, function as necessary to return the regulatory networks to the non-disease state. Therefore, future studies should be focused on improving our understanding of the basic regulatory networks for nucleic acid-sensing TLRs so that we may determine which are defective in SLE. Our hope is that these studies will lead to novel drug development, and improve our repertoire of options to treat autoimmune disease.

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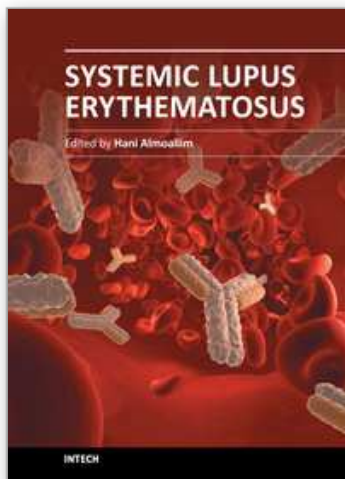
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This book provides a comprehensive overview of the basic and clinical sciences of Systemic Lupus Erythematosus. It is suitable for basic scientists looking for detailed coverage of their areas of interest. It describes how advances in molecular biology have increased our understanding of this disease. It is a valuable clinical resource for practicing clinicians from different disciplines including rheumatologists, rheumatology fellows and residents. This book provides convenient access to information you need about cytokines, genetics, Fas pathway, toll like receptors and atherogenesis in SLE. Animal models have been reviewed as well. How to avoid delay in SLE diagnosis and management, in addition to various clinical manifestations including pregnancy and SLE have all been explained thoroughly in this book.

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