Cutting Edge: A TLR9 Cytoplasmic Tyrosine Motif Is Selectively Required for Proinflammatory Cytokine Production

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Compartmentalization of nucleic acid sensing TLR9 has been implicated as a mechanism to prevent recognition of self nucleic acid structures. Furthermore, recognition of CpG DNA in different endosomal compartments leads to the production of the proinflammatory cytokine TNF-α, or type I IFN. We previously characterized a tyrosine-based motif at aa 888–891 in the cytoplasmic tail of TLR9 important for appropriate intracellular localization. In this article, we show that this motif is selectively required for the production of TNF, but not IFN. In response to CpG DNA stimulation, the proteolytically processed 80-kDa fragment is tyrosine phosphorylated. Although Y888 is not itself phosphorylated, the structure of this motif is necessary for both TLR9 phosphorylation and TNF-α production in response to CpG DNA. We conclude that bifurcation in TLR9 signaling is regulated by a critical tyrosine motif in the cytoplasmic tail.}

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Nucleic acid sensing TLRs are important for host defense against pathogens but are also potent immunomodulators. For example, the TLR9 ligand CpG DNA is an adjuvant for vaccines in nonhuman primates (1). CpG DNAs are also versatile, because depending on the sequence and chemical properties of the CpG DNA, TLR9 signaling can preferentially result in proinflammatory cytokine production and B cell proliferation (CpG DNA-B/D), or in type I IFN immune production (CpG DNA-A/K) (2, 3).

The outcome of response to the two different classes of CpG DNAs depends on the endosomal compartment where contact with TLR9 occurs (4). CpG DNA-A/D and CpG DNA B/K are endocytosed but then are preferentially retained in early endosomes (CpG DNA-A) to elicit IFN production or in lysosomes (CpG DNA-B) to elicit proinflammatory cytokines (4). TLR9 gains access to these DNAs by trafficking from the endoplasmic reticulum, through the Golgi (5–7). Recent data implicate adaptor protein 3 (AP3) in regulation of TLR9 trafficking from the Golgi to lysosome-related organelles where IFN production occurs, but whether AP3 is selectively required for IFN production or is also required for TNF production is controversial (8, 9). Regardless, distinct regulatory mechanisms selectively governing inflammatory cytokine production have not been identified.

We hypothesized that one of several discrete TLR9 localization motifs was phosphorylated and regulated signaling (YXXΦ, where Φ is any amino acid, Φ is a bulky hydrophobic amino acid) (10–12). We show that TLR9 with a single point mutation at Y888 (Y→A) selectively impairs TNF production and receptor phosphorylation. Mutation of Y888 to the structurally conserved phenylalanine (Y→F) retained TNF production and phosphorylation. We conclude that although not directly phosphorylated, Y888 was structurally required for phosphorylation of TLR9, and thereby regulates TLR9-mediated signal bifurcation.

Materials and Methods

Reagents and plasmids

The following Abs and reagents were used: hemagglutinin (HA; ABM and Roche), Rab5, phospho-p38 and total p38 (Cell Signaling Technologies), TNF-α ELISA kit (BioLegend), CpG DNA 2216 and 10104 (Eurofins MWG and Integrated DNA Technologies), LPS (Invivogen), and DOTAP (Roche). QuikChange (Stratagene) was used for site-directed mutagenesis of TLR9-HA. Primer sequences are available upon request.

Cell culture, retroviral transduction, and immunoblot analysis

TLR9−/− macrophages were cultured in DMEM with 10% (v/v) FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES (complete DMEM), and 10 μg/ml ciprofloxacin. CpG DNA–DOTAP complexes were prepared as previously described (9). Retroviral supernatant with polybrene (8 μg/ml) was used in spin transductions. Cells were cultured at 37°C for 44–48 h before stimulation. Immunoblotting was performed as previously described (7, 13).

Real-time PCR

RNA (QiAShredder and RNaseasy kit; Qiagen) was used to prepare cDNA (SuperScript III First-Strand Synthesis; Invitrogen). PCR was performed with

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SYBR Green Supermix (Applied Biosystems) on an ABI PRISM 7500 (Applied Biosystems). Changes in gene expression were determined using the 2^ΔΔCT method.

**Immunofluorescent staining**

TLR9^−/− macrophages were transduced before treatment with 3′ Cy3-10104 CpG DNA, 2216 CpG DNA in a complex with DOTAP or medium. Cells were stained for HA and CD107a or Rab5 followed by Alexa Fluor 488 and Alexa Fluor 647 Abs. Coverslips were mounted with Prolong Gold Antifade Reagent (Life Technologies) and visualized on a Leica TCS SP5 confocal microscope using a 63× oil objective (Leica Microsystems). Image analysis was performed with the colocalization tool. Figures were prepared in Photoshop (Adobe).

**Results and Discussion**

**A cytoplasmic tyrosine motif is required for TNF production**

We previously identified a highly conserved 4-aa motif in the cytoplasmic tail of TLR9 that contributes to intracellular trafficking (11). To determine whether this motif regulated production of the proinflammatory cytokine TNF, IFN, or both, we retrovirally transduced TLR9-deficient macrophages with wild-type (WT) TLR9, the tyrosine mutant (TLR9^Y888A), or empty vector, and assayed for TNF-α production in response to CpG DNA. Vector-transduced cells did not respond, whereas cells expressing WT TLR9 produced TNF-α in response to CpG DNA-B (Fig. 1A). However, cells expressing TLR9^Y888A produced no TNF-α in response to CpG DNA-B (Fig. 1A). LPS responses were similar in all cells (Fig. 1B).

We next asked whether IFN production was also compromised in cells expressing mutant TLR9. WT TLR9, but not empty vector, reconstituted cells responded to CpG DNA-A in complex with DOTAP to induce IFN-β mRNA (Fig. 1C). CpG DNA-A alone, CpG DNA-B alone, or CpG DNA-B in complex with DOTAP did not induce IFN (Fig. 1C; A.C. and C.A.L., unpublished observations). However, unlike for TNF production (Fig. 1A), TLR9^Y888A supported near WT levels of IFN-β mRNA induction (Fig. 1C). Together, these data showed that a specific tyrosine motif in the cytoplasmic tail of TLR9 was selectively required for production of TNF.

**TLR9 receptor phosphorylation correlates with TNF production and depends on Y888**

YXXΦ motifs can be phosphorylated, and thereby regulate signaling (14); furthermore, TLR9 is phosphorylated in response to CpG DNA stimulation (12). Stimulation with CpG DNA-B resulted in phosphorylation of an 80-kDa form corresponding to the mature receptor (Fig. 2A) (13, 15–18). Full-length TLR9 was not abundant in macrophages, so it is unclear whether the phosphorylation event occurs exclusively on p80 or also on full-length TLR9. Regardless, mutation of Y888 to alanine had no effect on proteolytic processing of TLR9 (Fig. 2A) but eliminated CpG DNA-dependent p80 phosphorylation (Fig. 2A), suggesting that TLR9 phosphorylation depended on Y888. Mutation of the critical tyrosine to phenylalanine, a structurally conserved but nonphosphorylated amino acid, did not block phosphorylation. Therefore, phosphorylation depends on the structural motif, not on phosphorylation of Y888. Mutation of any one tyrosine in the

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**FIGURE 1.** A tyrosine motif in TLR9 cytoplasmic domain is required for proinflammatory cytokine response. **A**, TLR9^−/− macrophages were retrovirally transduced with empty vector (Vector), WT TLR9, TLR9^Y888A (Y888A), or TLR9^Y888F (Y888F). Cells were stimulated with media or CpG DNA-B (10 μg/ml) for 8 h. Secreted TNF-α concentration was determined by ELISA. Error bars indicate SD (n = 4). **B**, as in A, except cells were stimulated with LPS (100 ng/ml). **C**, Vector, WT, or Y888A cells were stimulated with CpG DNA-A (10 μg/ml) or CpG DNA-B (10 μg/ml) in complex with DOTAP, and IFN-β mRNA relative expression level (REL%) was determined by real-time PCR. Results are representative of three experiments.

**FIGURE 2.** WT TLR9 and TLR9^Y888F, but not TLR9^Y888A, are tyrosine phosphorylated in response to CpG DNA and support downstream signaling. TLR9^−/− macrophages were retrovirally transduced with empty vector (V), TLR9-HA (WT), TLR9^Y888F_HA (Y888F), or TLR9^Y888A_HA (Y888A). Cells were stimulated with CpG DNA-B (10 μg/ml) for the indicated times. **A**, HA immunoprecipitates were immunoblotted for phosphotyrosine or HA. Arrow indicates full-length TLR9; asterisks indicate p80. **B**, Whole-cell lysates were immunoblotted for phospho-p38 and total p38. Representative of two experiments.
cytoplasmic tail of TLR9 does not abrogate phosphorylation (C.A.L., unpublished observations). TLR9 phosphorylation is complex and likely involves more than one site. WT TLR9, but not the Y888A mutant, supported downstream signaling as measured by time-dependent p38 phosphorylation in response to CpG DNA-B stimulation (Fig. 2B). Failure of TLR9Y888A to support TNF was not due to lack of binding to ligand, because TLR9Y888A in lysates interacted with CpG DNA-B as well as, or better than, WT TLR9 (A.C. and C.A.L., unpublished observations). Therefore, TLR9 phosphorylation occurred selectively on the p80 form of the receptor, depended on Y888, and correlated with TNF production.

The structure of Y888 is critical for TNF production

We next asked whether Y888 was directly phosphorylated by mutation of the critical tyrosine to phenylalanine, which is structurally related to tyrosine, but cannot be phosphorylated because of the lack of the hydroxyl group (19). When expressed in TLR9−/− macrophages, TLR9Y888F was proteolytically cleaved (Fig. 2A), and supported downstream signaling as indicated by CpG DNA-induced p38 phosphorylation (Fig. 2B) and TNF-α production in response to CpG DNA-B (Fig. 1A). LPS responses were similar regardless of the transduction conditions (Fig. 1B). Therefore, although Y888 was not directly phosphorylated, it was structurally required for TLR9 to support production of TNF.

Y888 is required for TLR9 trafficking to lysosomal-associated membrane protein 1-positive endolysosomes

We next asked whether the mechanism by which the Y888 motif selectively regulated cytokine production was through access to endolysosomes. CpG DNA is endocytosed to early endosomes within 5 min and traffics to late endosomes/endolysosomes at 1 h (4, 7). The endolysosomal compartment is where proinflammatory cytokine production initiates and is identified by the presence of lysosomal-associated membrane protein 1 (LAMP-1). WT and mutant TLR9 were similarly localized in untreated cells (A.C., W.R., and C.A.L., unpublished observations). However, after a 1-h incubation with 3′-Cy3–labeled CpG DNA, WT TLR9 and TLR9Y888F, but not TLR9Y888A, colocalized with LAMP-1 compartments as determined by four-color confocal microscopy (Fig. 3). Pearson’s coefficients calculated from line scan analyses from multiple sections of multiple cells (not shown) demonstrated colocalization of WT and the F mutant with CpG DNA (WT: 0.4949, TLR9Y888F: 0.6192) and LAMP-1 (WT: 0.7344, TLR9Y888F: 0.7015; Fig. 3). Pearson’s colocalization coefficients were significantly lower for TLR9Y888A with CpG DNA (0.1141) and LAMP-1 (0.2186). Together, these data show that TLR9Y888A fails to induce production of TNF because it fails to reach LAMP-1 endolysosomes.

Because TLR9Y888A supported IFN production, we asked whether TLR9Y888A localized with early endosome markers after stimulation with CpG DNA-A–DOTAP complexes. Within 10 min of exposure to CpG DNA-A–DOTAP complexes, both WT and TLR9Y888A colocalized with Rab5 (Fig. 4). Together with the observation that TLR9Y888A did not colocalize with LAMP-1 1 h after CpG DNA-B incubation,
we conclude that this structural motif is required to enable trafficking of TLR9 from early endosomes to late endosomes where proinflammatory signaling is initiated.

TLR9 signals to induce proinflammatory cytokines and IFN from different endosomal compartments. The mechanisms regulating this signal bifurcation are not fully understood, although they likely require AP3 (8, 9). In this study, we showed that a single tyrosine mutation in the cytoplasmic tail of TLR9 selectively impacts TNF production. Tyrosine phosphorylation of mature TLR9 correlated with proinflammatory cytokine production, but not IFN production, and depended on Y888. Our observation that mutation of Y888 to alanine inhibits TLR9 phosphorylation, yet selectively impacts only TNF production, seemingly contradicts observations by Sanjuan et al. (12). They showed that Src kinase inhibitors, such as PP2, reduce both TNF and IFN production, as well as inhibit tyrosine phosphorylation of TLR9. However, PP2 inhibition of IFN and TNF responses does not necessarily mean that blocking tyrosine phosphorylation of TLR9 itself inhibits both responses. AP3 has recently been implicated in regulation of TLR9 access to the IFN- and proinflammatory cytokine-inducing compartments (8, 9). Our motif likely does bind AP3 because IFN production was normal for TLR9Y888A. Thus, our data support a model where TLR9 does bind AP3 because IFN production was normal for TLR9Y888A. Our motif likely inhibits both responses. AP3 may have been implicated in regulation of TLR9 access to the IFN- and proinflammatory cytokine production. Together, these motifs, and the regulatory proteins that bind them, would be targets to therapeutically manipulate cellular response to CpG DNA.

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Disclosures
The authors have no financial conflicts of interest.

References


Corrections


In Fig. 1, the data for the Y888F mutant was omitted. These data are included in the updated Fig. 1 below. The figure legend was correct as published and is included here for reference.

**FIGURE 1.** A tyrosine motif in TLR9 cytoplasmic domain is required for proinflammatory cytokine response. A, TLR9−/− macrophages were retrovirally transduced with empty vector (Vector), WT TLR9, TLR9Y888A (Y888A), or TLR9Y888F (Y888F). Cells were stimulated with media or CpG DNA-B (10 μg/ml) for 8 h. Secreted TNF-α concentration was determined by ELISA. Error bars indicate SD (n = 4). B, As in A, except cells were stimulated with LPS (100 ng/ml). C, Vector, WT, or Y888A cells were stimulated with CpG DNA-A (10 μg/ml) or CpG DNA-B (10 μg/ml) in complex with DOTAP, and IFN-β mRNA relative expression level (REL%) was determined by real-time PCR. Results are representative of three experiments.

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