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TRADITIONAL BIOCHEMICAL ASSAYS FOR STUDYING TOLL-LIKE RECEPTOR 9

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Abstract

Understanding the mechanistic basis of receptor activation and regulation can offer therapeutic targets for disease treatment. Evidence is emerging for a role of the normally foreign responsive Toll-like receptors (TLRs) in the development of autoimmunity through response to self-patterns. Regulatory mechanisms governing this class of receptors are poorly understood, and failures within this system likely contribute to development of autoimmunity. In this article, we review biochemical assays used to study one of the self-pattern responsive TLRs, TLR9, and suggest that these studies are critical for development of new targets for autoimmune therapies.

Keywords

CpG DNA; innate immunity; Toll-like receptor 9

INTRODUCTION

Although DNA is the blueprint of life, it is also a very potent stimulus for our immune system. Detection of DNA by innate immune receptors is a warning sign for cell and tissue damage and initiates inflammatory and tissue repair processes. Synthetic DNA mimics the stimulatory activity of bacterial DNA and is being tested in cancer therapy and as a vaccine adjuvant. Unfortunately, inappropriate or uncontrolled response to self-DNA contributes to autoimmunity. To identify potential targets with immunotherapeutic potential in autoimmune disease, we must first understand the molecular mechanism of immune recognition of foreign DNA and the regulatory mechanisms that restrict response to host DNA. In this review, we will discuss cell biology and biochemical approaches that have been used to gain a better understanding of the regulation of the innate immune receptor for DNA, Toll-like receptor 9.

Toll-like receptors (TLR) are a class of innate immune response proteins that are germline encoded and recognize a wide array of microbial structures such as lipopolysaccharide, flagellin, lipopeptides, and nucleic acids including DNA.^[1] TLR9 recognizes bacterial, viral, and parasitic DNA via CpG motifs,^[2–11] and mice deficient in TLR9 lack responsiveness to DNA.^[10] Synthetic DNA containing CpG motifs (referred to hereafter as CpG DNA)

mimics the immunostimulatory activity of microbial DNA, and optimal CpG motifs with different stimulatory properties have been identified.^[12] Depending on the surrounding sequence and backbone chemistry, synthetic CpG DNAs have been synthesized that preferentially induce B cell and plasmacytoid dendritic cell (pDC) activation (class B, also known as K), induce high interferon- α production (class A, also known as D), or a combination of both response profiles (class C).^[13,14] There is some species specificity of CpG DNA response, suggesting co-evolution for optimal responses.^[9]

Under normal conditions, vertebrate DNA is not immunostimulatory due to CpG suppression,^[15] CpG methylation,^[16,17] and suppressive motifs.^[18,19] Despite these multiple levels of control, vertebrate DNA can stimulate immune cells through unmethylated CG-rich regions such as CpG islands in promoters.^[4,20,21] Breakdown of regulatory mechanisms that permit immune cell responses to vertebrate/self DNA likely contributes to autoimmune disease. Although the initiating events are unknown, systemic lupus erythematosus is an example where self-DNA in complex with anti-DNA antibodies synergizes to stimulate B cells.^[22] Interestingly, unlike most receptors, nucleic acid sensing TLRs are not found at the cell surface,^[11,23,24] and this unique localization may contribute to regulation of host DNA recognition.^[25]

In order for TLR9 to respond, DNA must be internalized. Physical inhibition of uptake, by covalently crosslinking CpG DNA to a bead, inhibits B cell responses to CpG.^[2,26] Cellular uptake of CpG DNA is mediated by caveolin-independent, receptor-mediated endocytosis.^[24,27] Covalent cross-linking of CpG DNA to antigen enhances uptake and presentation by antigen presenting cells, providing further evidence for receptor-mediated endocytosis.^[28,29] Despite intense effort, a surface receptor has not been identified.^[30–35] Regardless of the identity of the receptor, it is not CpG DNA specific, since non-CpG containing DNAs also enhance antigen uptake, and compete for CpG DNA uptake.^[36–38] Recent studies show that a chemokine (CXCL16) enhances cellular response to CpG DNA, but only of the A class.^[39] Future studies will likely identify other surface receptors that enhance DNA uptake.

Upon internalization, CpG DNA traffics to TLR9 and initiates signaling in an acidic endosomal compartment. Inhibitors of endosomal acidification block CpG DNA induced signaling without affecting endocytosis.^[40] Combined with the observation that TLR9 only binds to CpG DNA at pH 6.5 or lower, a model has emerged that TLR9 recognizes CpG DNA in an acidic endosome.^[41,42] TLR9 and CpG DNA co-localize in endosomes as determined by immunofluorescent microscopy.^[11,24,43,44] Interestingly, TLR9 is in the endoplasmic reticulum (ER) prior to cell stimulation,^[23,24] suggesting an induced trafficking event upon exposure to CpG DNA. In support of this, TLR9 is only biotinylated by a membrane impermeable sulfo-NHS-biotin reagent following CpG DNA stimulation.^[24] TLR9 is also only detected in detergent insoluble lipid raft cell fractions following CpG DNA stimulation.^[45] While it is clear that TLR9 traffics from the ER to acidic endosomes to recognize CpG DNA, the mechanisms by which this occurs are only partly understood. Here we will review some of the important biochemical and cell biological techniques that have led to major breakthroughs in our understanding of TLR9 biology. We suggest that these

basic studies have provided, and will continue to provide, key information necessary to develop mechanisms to manipulate signaling for therapeutic benefit.

MICROSCOPY STUDIES AND FUSION PROTEINS

Major discoveries about TLR9 localization and trafficking have come through studies using fusion proteins. An N-terminal fusion of hemagglutinin (HA) to human TLR9 followed by incubation with fluorescently labeled ligand permitted the first visualization of this TLR co-localizing with CpG DNA.^[11] Fusion of adapter proteins, such as MyD88, to fluorescent proteins demonstrated that LPS, the ligand for TLR4, induced MyD88 recruitment to the cell surface, which was consistent with TLR4 expression at the cell surface.^[43] In contrast, CpG DNA induced recruitment of MyD88 to punctate, endosome-like structures.^[43] Together, these studies were some of the first to support a model where TLR9 initiated signaling from intra-cellular vesicles. Direct fusion of fluorescent proteins to TLR9 (green fluorescent protein, GFP, and yellow fluorescent protein, YFP) permitted localization studies of TLR9 itself.^[23,24] While TLR4-YFP was readily detected at the cell surface, TLR9-YFP was not.^[23,24,46] An example from our lab is shown in Figure 1, where anti-TLR4 surface staining revealed prominent surface expression of TLR4-GFP, while anti-TLR9 surface staining failed to detect the expressed TLR9-GFP. Furthermore, co-localization analysis showed that, prior to stimulation, TLR9 was primarily localized in the endoplasmic reticulum.^[23,24] Localization studies showed similar results in pDC and HEK293 cells,^[24] which supported the use of heterologous cell lines for biochemical and cell biology studies. Follow-up studies have now indicated that this localization is biologically important, restricting TLR9 response to self-DNA and thus avoiding autoimmune inflammation.^[25] Altogether, studies with fusion proteins have provided key information about localization and initiation of signaling.

PULSE CHASE ANALYSIS

Traditional biochemical studies such as pulse chase analysis provide important information about the kinetics of protein maturation and turnover. While static analysis of protein localization can shed light on some aspects of protein biochemistry, analysis of a synchronized protein population allows for a more refined analysis. By pulsing cells with ³⁵S-methionine and examining the total protein at different time points, we showed that the half-life of TLR9 was about 8 hours.^[23] The half-life of TLR9 is indicative of a protein that is matured, traffics, and attempts to perform its function, and then is degraded. This is consistent with a small amount of TLR protein being maintained in cells and with the trafficking of a small amount of TLR9 to survey the endosomal compartment, as has been proposed.^[23,47] Using pulse chase analysis to examine the stability of TLR9 in macrophages led to the interesting finding that TLR9 was proteolytically processed to a mature form in endosomes.^[48,49] Furthermore, pulse chase analysis combined with glycosylation analysis (described below) has significantly advanced our understanding of regulatory mechanisms governing the intracellular localization of TLR9.^[25]

GLYCOSYLATION ANALYSIS

While microscopy studies are informative, they must be supported by more rigorous and quantitative biochemical analysis. A sensitive deglycosylation assay has been used to provide biochemical evidence to support the localization of TLR9 to the ER.^[23,24] Glycosylated proteins trafficking through the Golgi complex are processed from the high mannose forms present in the ER to hybrid and complex glycoproteins (Figure 2). In the ER, preassembled high mannose-capped glycans are transferred in bulk onto asparagines in the consensus sequence Asn-X-Ser/Thr. Following association with chaperones and proofreading for proper protein folding in the ER, most membrane and secreted proteins are trafficked to the Golgi complex where mannose residues are removed and N-acetylglucosamine residues are added. Endoglycosidase H recognizes high mannose carbohydrates and cleaves between the two N-acetylglucosamine residues in the core region (Figure 2). However, once the mannose residues are trimmed, i.e., once a protein enters the cis-Golgi, the protein is generally no longer sensitive to digestion with EndoH. These modified proteins remain sensitive to Peptide: N-Glycosidase F digestion. Glycan analysis of TLR4 demonstrated that some TLR4 is resistant to EndoH, consistent with surface and Golgi complex localization of TLR4.^[46,50] In contrast, TLR9 was completely sensitive to EndoH digestion.^[23,24] We, and others, have used this as supporting evidence for TLR9 localization in the ER. However, there may be cell type differences in glycosylation since, in macrophages, a small amount of mature glycosylated TLR9 is observed.^[25] Furthermore, EndoH is not a definitive marker for ER localization. Although EndoH digests both high mannose glycans (found in the ER) and hybrid glycan modifications (found in the Golgi complex),^[51] there are examples of proteins that traffic to the cell surface despite remaining sensitive to EndoH.^[52–54]

To overcome limitations of glycosidase analysis, we adapted a lectin-blotting assay to more precisely define the glycan modifications on TLR9. Lectins are plant proteins that are highly specific for recognition of protein carbohydrate modifications. *Galanthus nivalis* lectin (GNL) is specific for high mannose containing structures found in immature and hybrid carbohydrates, while *Datura stramonium* lectin (DSL) binds to hybrid and complex glycan structures found only on proteins that have trafficked to the Golgi. Using this approach, we demonstrated that GNL binds to both TLR9 and TLR4, as would be expected from N-linked glycosylated proteins.^[47] Somewhat surprisingly, DSL bound to TLR4 and TLR9 indicating that both proteins reached the Golgi.^[47] Since GNL binds only to high mannose or hybrid glycans, digestion with EndoH eliminated binding of GNL to either TLR4 or TLR9. Interestingly, EndoH digestion eliminated DSL binding to TLR9, but did not affect DSL binding to TLR4. This indicated that the hybrid carbohydrates associated with TLR9 remained sensitive to EndoH, while those of TLR4 were resistant. Since this assay more precisely defined TLR9 glycosylations, lectin blotting is superior to EndoH digestion. This assay revealed that TLR9 constitutively traffics through the Golgi.

FURIN PROTEOLYTIC CLEAVAGE ASSAY

To track TLR9 trafficking through the trans-Golgi, we have developed an assay based on the cleavage of an N-terminal hemagglutinin (HA) tag by furin. Furin is a peptidase localized in

the trans-Golgi network (TGN) and at the cell surface.^[55–57] Therefore, only proteins that have trafficked at least as far as the TGN are cleaved by furin. TLR9 does not contain a furin cleavage site and is not normally cleaved by furin. We engineered a HA-tagged TLR9 with a furin cleavage site between the tag and TLR9 (HA^{fu}-TLR9). When wild type HA-TLR9 or HA^{fu}-TLR9 was immunoprecipitated and treated *in vitro* with recombinant furin, total TLR9 levels did not change, but furin cleaved the HA tag from HA^{fu}-TLR9 demonstrating the utility of this assay for detecting exposure of TLR9 to furin.^[47] By parallel immunoblot analysis with either an HA antibody, or a TLR9 antibody, we discovered that a small amount of HA^{fu}-TLR9 lost the HA tag, indicating that this fusion protein was exposed to furin under normal conditions. We concluded that some TLR9 had already trafficked out of the ER and into the Golgi prior to ligand addition.

ORGANELLE FRACTIONATION

A robust biochemical strategy for determining localization of a protein is organelle fractionation. Density gradient centrifugation over self-forming Percoll-sucrose gradients allows the separation of organelles based on density. Lysosomes, which are dense, migrate to the bottom of the gradient, while ER migrates to the middle and early endosomes are found in the lowest density fractions. Unloading gradients from the top, resolving each fraction side-by-side using SDS-PAGE, and performing immunoblot analysis for different organelle markers, allows identification of various cellular compartments. TLR9 was observed in LAMP-1 positive fractions, and immunoprecipitation studies demonstrated that this form had glycan modifications indicative of Golgi transit.^[47] This provided strong biochemical support for TLR9 trafficking constitutively through the Golgi and endosomal compartments.

CHIMERIC PROTEIN FUSIONS AND TRUNCATION ANALYSES

While the previous outlined studies showed TLR9 localization and trafficking, further molecular and biochemical analysis was required to define the regulatory mechanisms governing this localization and trafficking. TLR cytoplasmic domains have been fused to CD4, the IL-2 receptor alpha chain, TLR4, or integrins.^[58–61] When the transmembrane and TIR domain of TLR9 was fused to TLR4, integrin, or the IL-2 receptor alpha chain, the resulting chimera was not expressed at the cell surface. Similar results were obtained with other nucleic acid sensing TLRs, while surface TLRs dictated surface localization for fusion proteins. The transmembrane domain of mouse TLR9 also induced intracellular localization of mouse TLR4, suggesting that intracellular trafficking was regulated by the transmembrane domain. However, fusion of four to 52 amino acids of the cytoplasmic tail plus the transmembrane domain of human TLR9 to the IL-2 receptor alpha chain resulted in a protein that localized to the cell surface. This study ruled out a role for the transmembrane domain. It is unclear why two chimeras identified different regulatory domains. It could be attributed to different species of TLR9, however, our lab has observed similar regulatory mechanisms between human and mouse TLR9.^[44,61] We identified a 14 amino acid motif from the cytoplasmic tail of TLR9 that regulated intracellular trafficking,^[61] and TLR4 containing only the 14 amino acid motif of TLR9 mimics the localization of TLR9 suggesting this motif controls localization.^[61] The motif contains the sequence YNEL that is

similar to tyrosine-based localization motifs.^[62,63] Tyrosine-based localization motifs require a tyrosine followed by a hydrophobic amino acid at the +3 position, YXXΦ (X is any amino acid). More recently, this 14 amino acid motif has been shown to selectively regulate cytokine production through regulation of endosomal trafficking.^[44,61] Substitution of tyrosine at amino acid 888 for phenylalanine resulted in a protein that could induce interferon responses that were normal, but was selectively defective in TNF production.^[44]

POST-TRANSLATIONAL MODIFICATION: PHOSPHORYLATION

Recent studies have shown that TLR9, and several other TLRs (3, 4, 5, and 8), are tyrosine phosphorylated following ligand binding.^[44,64–68] We showed that phosphorylation of tyrosine (Y888) in the cytoplasmic tail of TLR9 occurs within a YXXΦ motif.^[44] Mutation of Y888 to either alanine or phenylalanine eliminated the ability of CpG DNA to induce TNF production from macrophages.^[44] Interestingly, in TLR9 knockout macrophages reconstituted with wild type or mutant TLR9, neither tyrosine phosphorylation nor structure of Y888 within the YXXΦ motif was necessary for type I interferon production.^[44] Similar to TLR9, most TLRs contain several cytoplasmic tyrosine residues, and phosphorylation of specific tyrosines within YXXΦ motifs was necessary for pro-inflammatory cytokine production.^[44,64–68] As with TLR9, mutation of tyrosines to phenylalanines in TLR3 (Y759), or TLR8 (Y1048), demonstrated that induction of the IFN pathway was independent of phosphorylation of the YXXΦ motif.^[67–69]

While the kinases responsible for TLR tyrosine phosphorylation remain unknown, several members of the Src family kinases have been implicated.^[64–67] In support of a role for Src family kinases in TLR9 signaling, tyrosine phosphorylation inhibitors such as PP1 and PP2 reduced pro-inflammatory cytokine production.^[66] However, at least for LPS-mediated TLR4 signaling, macrophages derived from Src family Lyn/Hck/Fgr triple knockout mice showed normal pro-inflammatory cytokine production,^[70] indicating that another Src family kinase is responsible or a dynamic contribution of each kinase is required.^[71] Btk, a Tec family kinase, interacts with the cytoplasmic domain of a number of TLRs including TLR9 by yeast two-hybrid screening.^[72] Furthermore, following ligand stimulation, Btk co-immunoprecipitated with TLR8 and TLR9 in THP-1 monocytic cells.^[73] Interestingly, X-linked agammaglobulinaemia patients, who lack functional Btk, showed reduced induction of IL-6 following CpG-B DNA stimulation.^[73] The exact role of Btk is yet unclear since it also phosphorylates Mal, an adaptor molecule for TLR2 and TLR4.^[73,74] The YXXΦ motif, which Btk phosphorylates on Mal, is associated with PI3 K-dependent endosomal trafficking.^[73] Therefore, Btk activity could regulate TLR trafficking to endosomes where induction of the pro-inflammatory cytokine signaling cascade occurs. Collectively, phosphorylation of tyrosine within the YXXΦ motif in the cytoplasmic domain plays an important role in regulating TLR signaling. Further mutational analysis will elucidate the precise role of post-translational modifications in TLR signaling and trafficking. One caveat to these studies is that regulation of TLR phosphorylation and signaling may vary dependent upon cell type (Chockalingam et al., unpublished observations), adding further complexity to the system.

RETROVIRAL TRANSDUCTION METHODS FOR STUDYING TLR9 FUNCTION

The ability to transfer genetic information into cells has been paramount to our understanding of molecular and biochemical cellular processes. Traditional methods for ectopically expressing genes have included transfection using chemically precipitated DNA or by delivering nucleic acids within asymmetrically-charged lipid structures.^[75–77] More recently, investigators have taken advantage of viral delivery, nature's own evolutionary strategy for transferring genes into cells. Retroviral vectors, such as those derived from the murine leukemia virus,^[78] are effective tools for introducing genetic material into proliferating primary hematopoietic cells (Figure 3). This approach has allowed studies to be performed in relevant cell types that were previously intractable to genetic manipulation.

Ectopic expression and complementation of TLR9 function in macrophage cell lines (RAW 264.7 cells), and primary bone marrow-derived macrophage and dendritic cells from TLR9 deficient mice have yielded significant new information about the biology and regulation of this receptor. For example, the proteolytic events that regulate TLR9 function were identified by retrovirally transducing C-terminally tagged TLR9 into RAW 264.7 cells. This approach was necessary since the antibodies to mouse TLR9 do not specifically identify mouse TLR9, and TLR9 is proteolytically processed only in macrophages, not in HEK 293 cells.^[48,49,79] Retroviral reconstitution of TLR9 deficient macrophages was also used to demonstrate that a cytoplasmic localization motif of TLR9 selectively regulated proinflammatory cytokine production.^[44] Although B cells have not been extensively developed as a reconstitution system, retroviral manipulation of a B cell line revealed a role for the chaperones gp96 and CNPY3 in regulation of TLR9 folding.^[80] Recently, the use of retroviral vectors has been extended to *in vivo* by retrovirally transducing bone marrow stem cells with wild-type or mutant TLR9, a method call retrogenics. The retro-virally transduced stem cells repopulate TLR9-deficient mice, and introduction of a membrane mutant of TLR9 leads to increased serum DNA abundance and subsequent autoimmunity.^[81] The utility of both *in vitro* and *in vivo* reconstitution will dramatically advance the study of TLR9 regulation.

CONCLUSION AND OUTLOOK

The immune system is a complex interplay between cells that receive and distribute cues for initiation and regulation of responses. In general, immunologists try to understand the system as a whole, using disease models and genetically deficient animals to unravel the complexity. However, key advances in our understanding are often made using a reductionist approach, studying individual cells or individual proteins (cytokines, receptors, etc.). Cell biology and biochemistry approaches are necessary for these fundamental advances and provide concrete targets for therapeutic intervention. For example, while microarray studies provide a wealth of information on gene regulation, much of protein function is regulated post-translationally. We, and others, have capitalized on defining post-translational regulatory pathways for TLR9 to reveal key targets for therapeutic modulation. Other TLRs have the potential to cause autoimmune disease, and some of our discoveries are likely to be broadly applicable. However, some regulatory mechanisms appear highly

specific to one or a few TLRs. This offers selectivity for drug targeting, but necessitates additional studies to provide a detailed landscape for understanding the complexity of innate immune responses.

We envision development of specific drugs that modulate the function of distinct TLRs, reducing their unwanted hyperinflammatory activity in inflammatory diseases, such as autoimmunity. Because there are many TLRs that can combinatorially elicit protective immunity, blocking one would likely lead to compensatory mechanisms and not enhance susceptibility to infection. Just like tailoring of immunomodulators for improved immune function, TLRs, and potentially other innate immune receptors, could be selectively modulated to reduce their contribution to inflammatory disease. However, to achieve these goals and to advance the field of immunology, we must complement whole animal studies with basic biochemical studies.

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FIGURE 1.

Localization of human TLR9 and human TLR4. HeLa cells were transfected directly on coverslips with GFP-tagged TLR4 (top) or TLR9 (bottom). After 24 h, the cells were cooled on ice and washed in Hanks buffered salt solution with 0.1% bovine serum albumin and 0.1% azide. Cells were then stained, while live, with anti-TLR4 or anti-TLR9. The cells were fixed, counterstained with DAPI, and mounted on slides. Images were acquired with a Zeiss LSM510 meta, and assembled with Photoshop CS5 (Adobe Systems Inc.). Smaller black and white images show surface staining (anti-TLR4 or anti-TLR9) and total expression (TLR4-GFP or TLR9-GFP). Larger image shows the overlay of surface TLR (red), total TLR (green), and DNA (blue). Performed by C. Leifer (color figure available online).

FIGURE 2.

Analysis of TLR9 localization through glycosylation. High mannose glycan complexes are attached to asparagines in the endoplasmic reticulum. These complexes can be removed by either the EndoH glycosidase or the PNGase F glycosidase as indicated with the arrows (left). Once proteins reach the Golgi complex, the high mannose residues are trimmed and modified, resulting in hybrid or complex glycans. These complexes are not removed by EndoH, but are only removed by PNGase F (right). This digestion distinction permits rudimentary analysis of trafficking of N-link glycosylated proteins.

FIGURE 3.

Retroviral reconstitution of primary cells. Replication-deficient retroviral DNA vectors can be used to introduce a gene of interest into primary cells for studying TLRs in *in vitro* and *in vivo* models. Long terminal repeats (LTR) within the retroviral vector facilitate integration into the host genome, while an internal ribosomal entry site (IRES) and fluorescent marker gene, such as GFP, downstream of the gene of interest is helpful for tracking retroviral transduction of cells. Retroviral vectors are transfected into packaging cell lines, such as Phoenix cells, which express viral envelope and polymerase genes in trans, and viral particles are released into the cell culture media (supernatant). These retrovirus-containing supernatants are used to transfer DNA into hard-to-transfect cell lines, or primary cells such as bone marrow-derived macrophages, dendritic cells, or even stem cells. TLRs can then be examined in GFP⁺ cells in cell cultures (*in vitro*), or GFP⁺ stem cells can be transferred into irradiated-recipient mice (retrogenic mice) to repopulate gene-deficient mice *in vivo* (color figure available online).