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Review

Chemical Tools for Studying TLR Signaling Dynamics

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The detection of infectious pathogens is essential for the induction of antimicrobial immune responses. The innate immune system detects a wide array of microbes using a limited set of pattern-recognition receptors (PRRs). One family of PRRs with a central role in innate immunity are the Toll-like receptors (TLRs). Upon ligation, these receptors initiate signaling pathways culminating in the release of pro-inflammatory cytokines and/or type I interferons (IFN-I). In recent years, it has become evident that the specific subcellular location and timing of TLR activation affect signaling outcome. The subtlety of this signaling has led to a growing demand for chemical tools that provide the ability to conditionally control TLR activation. In this review, we survey current models for TLR signaling in time and space, discuss how chemical tools have contributed to our understanding of TLR ligands, and describe how they can aid further elucidation of the dynamic aspects of TLR signaling.

Innate Immune Signaling via Toll-like Receptors

Host defense against infection critically depends on the innate immune system, which recognizes invading pathogens through pattern-recognition receptors (PRRs). PRRs sense pathogens through the recognition of conserved pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002) and they recognize inappropriate cell death through the detection of danger-associated molecular patterns (McCarthy et al., 2014). Toll-like receptors (TLRs) are a key family of PRRs. Binding of conserved microbial structures, such as cell wall components, to TLRs results in the induction of a variety of signaling pathways. The outcome of these signaling events is the induction of pro-inflammatory cytokines, which act as general mobilizers of innate and adaptive immune cells, and/or type I interferons (IFN-I), inducing processes that directly inhibit microbial replication. Together these effector molecules lead to the immediate mobilization of both innate and adaptive immune components to appropriately combat the infectious agent. Recent developments in the study of TLR signaling have shed light on the complex orchestration of signaling events leading to IFN-I or pro-inflammatory cytokine production. This review focuses on this dynamic picture of TLR signaling as well as the chemical reagents currently developed to further elucidate temporal and spatial parameters in this pathway.

The mammalian TLR family consists of 13 members, of which TLR1 to TLR9 are conserved between humans and mice (Rehli, 2002). Due to a retroviral insertion, mouse TLR10 is not functional, whereas the human genome has lost TLR11, TLR12, and TLR13. All TLR family members share a common structure, consisting of an N-terminal ectodomain, a single transmembrane domain, and a C-terminal cytosolic Toll-interleukin-1 receptor (TIR) domain (Botos et al., 2011). The ectodomain contains multiple leucine-rich repeats that are involved in ligand recognition, whereas the TIR domain mediates recruitment of TIR domain-containing signaling proteins (Jenkins and Mansell, 2010). TLRs may be classified based on their predominant cellular localization in the absence of stimulation: TLR1, TLR2, TLR4, TLR5, and TLR6 mainly reside on the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 are retained intracellularly (Figure 1) (McGettrick and O'Neill, 2010).

Upon activation, TLRs dimerize (Botos et al., 2011). Most TLRs form homodimers, except for TLR2 which preferentially forms heterodimers with TLR1 or TLR6 (Kang et al., 2009; Jin et al., 2007). This initiates distinct signal transduction pathways that culminate in the transcription of genes important for host defense, including those coding for pro-inflammatory cytokines (e.g., tumor necrosis factor alpha [TNF-α], interleukin-6 [IL-6], IL-8) and type I interferons (e.g., IFN-α and IFN-β) (Tseng et al., 2010). Prototypically, the induction of pro-inflammatory cytokines is mediated through the transcription factor nuclear factor κB (NF-κB) (Li and Verma, 2002), whereas IFN-I transcription is induced by activation of members of the interferon regulatory transcription factor (IRF) family (Honda and Taniguchi, 2006). Pro-inflammatory cytokines and chemokines induce maturation of innate immune cells and help orchestrate subsequent adaptive immune responses. IFN-I interacts with the IFN-α/β receptor in an autocrine and paracrine manner, inducing the transcription of hundreds of IFN-stimulated genes (ISGs). Many ISGs harbor direct antimicrobial activities, particularly against viruses.

From the plasma membrane, TLRs survey cell surroundings for the presence of pathogens. For example, surface TLR2 and TLR4 can detect the bacterial cell wall components, peptidoglycans and glycolipids, from Gram-positive, and lipopolysaccharide (LPS) from Gram-negative bacteria, respectively (Mancini et al., 2014). TLR2 also detects viral structural proteins, despite their general variability (Barton, 2007; Thompson and Iwasaki, 2008). TLR5 detects flagellin from a wide range of bacterial species. Flagellin contains highly conserved sequence stretches critical

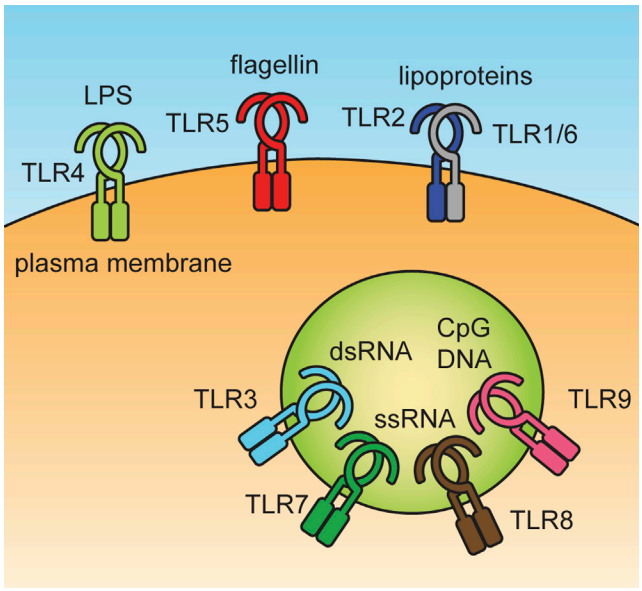

Figure 1. TLRs Can Be Classified Based on Their Predominant Cellular Localization in the Absence of Stimulation
TLR1, TLR2, TLR4, TLR5, and TLR6 mainly reside at the cell surface, whereas TLR3, TLR7, TLR8, and TLR9 are retained intracellularly. Examples of prototypical ligands are indicated.

for the execution of their motor function, thereby preventing the facile formation of escape mutants (Hayashi et al., 2001).

The intracellular TLRs (TLR3, TLR7, TLR8, and TLR9) sense PAMPs of pathogens that have entered cells. Viruses and bacteria typically enter cells through endocytosis or phagocytosis, and intracellular TLRs signal solely from vesicles along this pathway. Each TLR engages a different type of microbial nucleic acid (NA): TLR3 recognizes viral double-stranded RNA (Alexopoulou et al., 2001), TLR7 and TLR8 engage viral single-stranded RNA (Lund et al., 2004; Heil et al., 2004), and TLR9 recognizes unmethylated DNA containing CpG motifs (Hemmi et al., 2000). Restricting the activation of NA-sensing TLRs to endolysosomal vesicles is believed to serve as a mechanism to prevent the recognition of host-derived NA (Lee and Barton, 2014).

Recent studies have revealed additional layers of complexity related to the signaling of TLRs. For example, surface-expressed TLRs were shown to also be capable of signaling from intracellular vesicles (McGettrick and O'Neill, 2010). Furthermore, this signaling resulted in the production of different cytokine-expression profiles (Tan and Kagan, 2016). The finding that TLR4 induces NF-κB and IRF activation from different locations within the cell was the first example of how spatial context affects TLR signaling output (Figure 4A). This is also the case for the intracellular TLRs. Data suggest that they too signal differently from different intracellular compartments.

In this review, we discuss this emerging view that signal transduction via TLRs is orchestrated in space and time within cells, to induce a response of appropriate quality (specificity, amplitude, and duration). The importance of understanding the regulatory mechanisms is underscored by the fact that activation of mislocalized TLRs is implicated in recurrent viral infections (Blasius et al., 2010) and autoimmune diseases (Barton et al., 2006). Furthermore, we discuss the development of new reagents that allow the study of TLR signaling dynamics and can contribute to the delineation of spatial and temporal components of their signaling pathways.

Synthetic TLR Ligands

Chemists have made a major contribution to the elucidation of TLR function by developing selective synthetic TLR ligands (Table 1). For over a hundred years, it has been known that mixtures of attenuated bacteria, such as Coley's toxin, aid in the treatment of diseases such as cancer, through the induction of a general pro-inflammatory state (Hennessy et al., 2010). Yet, even after the identification of TLRs and other PRRs, it proved difficult to elucidate which receptor was responsible for detecting which PAMP. Strategies to extract natural ligands from complex biological samples helped in the discrimination of natural ligands for PRRs. The yeast cell wall extract zymosan is an example of a complex mixture, capable of activating both TLR2 and the β-glucan-binding receptor Dectin-1. Activation of TLR2 was thought to rely on lipopeptides in this extract. This was confirmed by extracting the lipopeptides using organic solvents, leaving a β-glucan preparation incapable of activating TLR2 (Ikeda et al., 2008). Characterization of natural ligands is further complicated by macromolecules that can engage multiple PRRs. A recent report from the Kawasaki laboratory showed that certain types of bacterial LPSs serve as dual PRR ligands (Wittmann et al., 2016). The lipid A portion of the LPS engages TLR4 via interaction with multiple fatty acid tails that are pendant from a di-glucosamine sugar. Additional sugars attached to the glucosaminyl-core can ligate to a second unrelated PRR, Dectin-2, through classical lectin-glycan interactions. This second interaction appears to modulate signaling from the lipid A/TLR4 interaction, thereby altering the overall immunological outcome. This Dectin-2 activation could be prevented by enzymatic removal of the extended glycan.

The study of TLR ligands has long been hampered by such complicating factors relating to PRR crosstalk. The elucidation of specific ligand-receptor pairs was difficult due to the absence of pure, well-defined ligands. This is where chemists made their first valuable contribution to the field: by developing defined synthetic ligands, specific TLRs could be activated selectively. This has also facilitated regulatory approval for their inclusion in vaccines and pro-inflammatory therapies. With great success, as dozens of synthetic TLR ligands are currently in clinical trials for the treatment of cancer, viral and bacterial infections, allergy, asthma, and autoimmune diseases (Hennessy et al., 2010). To date, two synthetic ligands have been approved by the FDA for clinical use: imiquimod (TLR7/8) is used as a 5% cream for human papillomavirus (HPV)-induced genital warts, actinic keratosis, and superficial basal cell carcinoma, and monophosphoryl lipid A (TLR4) is used as a vaccine adjuvant for HPV type 16 and type 18 (HPV16 and HPV18) (Vacchelli et al., 2012).

A great example of how chemistry, in combination with structural biology, has helped delineate TLR ligand interactions, is that of TLR2 binding its synthetic lipopeptide ligands. Pam_3CSK_4 (**1**) (Figure 2) was among the first discovered lipopeptides that acts as a lipoprotein mimic in activating TLR2/1 signaling pathways (Aliprantis et al., 1999) (Table 1). Pam_3CSK_4 is still a rather large molecule, containing three highly lipophilic tails and six amino acids. Via multiple iterations, this ligand has been

Table 1. Discovery of TLR Ligands Used for Caging Strategies

Toll-Like Receptor	Ligand(s)	Mechanism(s) of Action	References
TLR2	short synthetic lipopeptides with a palmitylated N-terminal amino acid: Pam_3CSK_4 and Pam_2CSK_4	mimic the immunostimulatory effect of bacterial lipopeptides	Norgard et al. (1996) Hoffmann et al. (1988) Aliprantis et al. (1999)
TLR4	non-lipid-like molecules structurally unrelated to known natural ligands: pyrimido[5,4-b]-indoles	likely dock in the LPS-binding pocket of TLR4	Chan et al. (2013)
TLR7 and TLR8	imidazoquinolines resembling the nucleoside guanosine: resiquimod (TLR7 and TLR8) and imiquimod (TLR7)	bind to ligand binding site, activate TLR7 and/or TLR8, and induce IFN-α in various cell types	Hemmi et al. (2002) Jurk et al. (2002) Shibata et al. (2016) Tanji et al. (2015)
TLR9	synthetic phosphorothioate-stabilized oligodeoxynucleotides (ODNs) with varying the number and location of CpG dimers and their overall nucleotide sequence	bind to TLR9, a sensor of CpG DNA, and lead to immunostimulatory effect mimicking effects of bacterial DNA	Krieg et al. (1995) Hemmi et al. (2000)

To date chemical caging strategies have been reported for five different synthetic TLR ligands. These ligands were discovered in various ways. Some mimic PAMPs present in pathogens, while others are structurally unrelated to natural ligands.

simplified by controlling structure and solubility, while retaining nanomolar activity. The palmitoyl tail present on the N terminus of the cysteine proved to be a dispensable moiety for TLR2 activation. Removal of this lipophilic tail resulted in the highly potent Pam_2CSK_4 (Mühlradt et al., 1998) (**2**) and introduced a preference for TLR2/6 heterodimerization (Takeuchi et al., 2002). The preference of TLR2 for either TLR1 or TLR6 heterodimerization is best explained with the help of the crystal structures of ligand-bound mouse TLR2/6 (Figure 3) and human TLR2/1 heterodimers (Jin et al., 2007). The two palmitoyl tails of the TLR ligand present on the glycerol moiety interact with TLR2 by strongly binding in a hydrophobic pocket, the volume of this pocket being 15% larger than theoretically necessary for binding. This surplus of space allows for flexibility in the lipid moieties when modifying the chemical structure (Buwitt-Beckmann et al., 2005). Dimerization of the now ligand-bound TLR2 can either occur with TLR1 or TLR6, depending on the presence of a lipid moiety on the N terminus of the cysteine. In the case of Pam_3CSK_4, the amide-bound palmitoyl tail is guided into a channel present in TLR1 by a hydrophobic wall formed by residues F312, F314, I319, and Y320, facilitating dimerization. In the Pam_2CSK_4 construct, however, the amide-bound lipid tail is lacking and this initiates a structural rearrangement of the hydrophobic wall. The corresponding residues of this wall in TLR6 move inside the pocket by means of hydrophobic interaction. This, in turn, pushes F317 out of the lipid channel, shifting the LRR11 loop in such a way that F319 can now engage in a hydrogen bond with the amide connecting the serine with the cysteine of the Pam_2CSK_4 construct. This hydrogen bond is absent in the Pam_3CSK_4-bound TLR2/1 crystal structure (Jin et al., 2007). Finally, TLR2/6 dimerization by Pam_3CSK_4 is prevented due to the hydrophobic channel on TLR6 being blocked by bulky side chains present on F343 and F365. Removing the bulky aromatic side chains through mutagenesis in TLR6 led to TLR2/6 dimerization by Pam_3CSK_4.

Using Pam_2CSK_4 as a benchmark, several structure-activity relationship (SAR) studies have been carried out (Figures 2 and 3B). The Cys-Ser lipodipeptide (**3**) was identified as the minimal structure necessary for TLR2 activation (Prass et al., 1987). Removal of serine yields an inactive Cys-OH monomer (Bessler et al., 1985). Replacing the amino acid by glycine (**4**), for instance, has little impact on the activity (Takeuchi et al., 2002). The stereocenter in the glycerol derivative cannot be inverted, as the absolute S-configuration shows complete abrogation of activity (**5**) (Metzger et al., 1991; Takeuchi et al., 2000). The configuration of the stereocenter on the dipeptide unit is not of absolute importance, as long as the carbonyl of the Cys-Ser amide bond can engage in H-bonding in the binding pocket of TLR2 (Wu et al., 2010b). Replacement of the thioether with an ether bridge (**6**) reduced the activity by eight orders of magnitude (Wu et al., 2010b), while replacement with a selenoether bridge (**7**) caused no difference in activity when compared with compound (**3**) (Agnihotri et al., 2011). Removal of a palmitoyl ester and a methylene yielded the simpler monoacylated PamCS methyl ester lipopeptide (**8**). This modification completely abrogated its activity as a murine TLR2 activator, while retaining the capacity to induce human TLR2 activation (Agnihotri et al., 2011). Compared with compound (**3**), compound (**8**) is both easier to synthesize and more water soluble (Agnihotri et al., 2011). Although acetylation of the cysteine amine (**9**) led to only a small enhancement of the agonistic activity on the receptor itself, the resulting cytokine production (after NF-κB nuclear translocation) was far greater at equal doses. The potency of this PamCS (**9**) approached that of Pam_2CS (**3**) (Salunke et al., 2012).

Although this methodology of finding superior ligands proved fruitful, it remains a process of trial and error. The recent spate of crystal structures of ligand-engaged TLRs and computer modeling studies will likely contribute greatly to the design of more selective, simpler, and more potent ligands (Botos et al., 2011).

Using Synthetic Ligands to Elucidate TLR Signaling

Well-defined and pure synthetic TLR ligands are used to study members of the TLR family in a broad spectrum of experimental settings and cell types, largely focusing on cell lines. Since good antibodies against many TLRs are lacking, recombinantly tagged TLR proteins have been introduced in cell lines, to allow detection with antibodies directed against the tag. Fluorescent

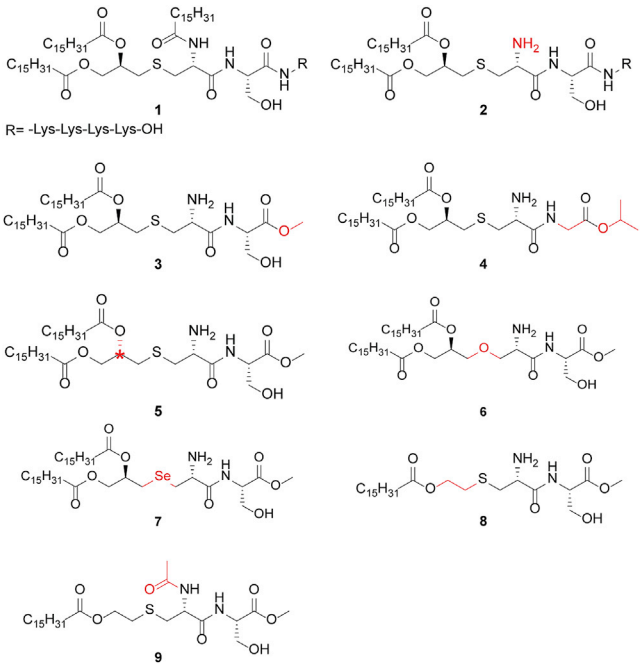

Figure 2. Structures and Relative Activities of Synthetic TLR2 Ligands

Depicted are TLR2 agonists that have resulted from several SAR studies. These studies aimed to simplify the ligand structure, while maintaining their activity and increasing their solubility in aqueous medium. Highlighted in red are alterations applied within the molecule. Relative activities (R.A.) are based on EC_{50} and IC_{50} values as reported in the biological evaluation of the respective compound in the cited study are **1**, **2** > **3**, **4**, **7**, **9** > **8** ≫ **6**; relative activity of **5** is unknown. First reports of structures: **1** (Aliprantis et al., 1999); **2** (Mühlradt et al., 1998); **3** (Farhat et al., 2008); **4**, **7**, **8** (Agnihotri et al., 2011); **5** (Takeuchi et al., 2000); **6** (Wu et al., 2010b); **9** (Salunke et al., 2012).

tags have also been applied for visualization by microscopy in living cells. It is, however, difficult to assess if the tags affect receptor trafficking and function. The delicacy of TLR signaling results in cell type-specific outcomes, and therefore immune responses induced by TLR activation should be addressed in their native cellular context (Wu et al., 2010a). A prominent example of cell type-specific outcomes of TLR activation is that of two subsets of dendritic cells (DCs). While a minor subset of DCs, the plasmacytoid DCs (pDCs), produces a large amount of IFN-I in response to TLR7 and TLR9 ligands, the conventional DCs (cDCs) mainly produce pro-inflammatory cytokines (Reizis et al., 2011; O'Keeffe et al., 2005).

In the above-mentioned settings, selective synthetic TLR ligands are frequently used to study receptor function. Detailed characterization of the synthetic ligands is of utmost importance, as small changes in ligand structures can affect the interaction with TLRs. Caution should be taken when translating functional results obtained with synthetic TLR ligands to a more natural mode of activation. As an example, small-molecule imidazoquinoline ligands (Table 1) and natural single-stranded RNA ligands occupy different binding pockets within TLR7 and TLR8, thereby inducing different functional outcomes (Zhang et al., 2016b; Colak et al., 2014).

In the past, research has primarily focused on TLR7. Differences in the molecular basis for ligand recognition and activation between TLR7 and TLR8 have recently led to a growing popularity for studying TLR8 function. This has culminated in a crystal structure of the imidazoquinoline ligand resiquimod in complex with human TLR8 (Tanji et al., 2013). From this structure, the mechanism by which resiquimod activates TLR8, and possibly TLR7, has been resolved. In solution, recombinant TLR8 proteins exist as dimers in an inactive form. Ligand binding induces a set of conformational changes leading to shortening of the gap between the C termini from 53 to 30 Å. This proximity allows dimerization of the TIR domains, thus leading to downstream signaling. Per dimer, two small-molecule ligands are able to bind due to a 2-fold symmetry. For the ligand resiquimod, the most notable hydrophilic interaction is with the carboxylate functionality in the side chain of an aspartic acid residue, which forms hydrogen bonds with the amidine functionality of the ligand in almost perfect geometry.

In recent years, chemists have started to develop more advanced chemical tools based on synthetic TLR ligands. For example, bi- and trifunctional TLR ligands have been developed. These ligands induce ligation of multiple TLRs in near proximity on the plasma membrane (Tom et al., 2015), and signaling output proved to be cumulative (Ryu et al., 2016). Recent data suggest that not only the cell type, but also the place and time of TLR activation within cells, critically affects signaling outcome. The spatiotemporal aspects of TLR signaling and novel chemical tools that may help delineate underlying regulatory mechanisms, are discussed below in more detail.

Surface-Expressed TLRs Induce NF-κB and IRF Activation from Different Locations within Cells

The first example of spatiotemporal control came from the study of TLR4 (Figure 4A). The signaling events leading to induction of pro-inflammatory cytokines originate from TLR4 ligation at the plasma membrane. Upon engagement of a ligand at the cell surface, TLR4 dimers recruit the sorting adaptor TIR domain-containing adaptor protein (TIRAP) and the signaling adaptor myeloid differentiation primary response 88 (MyD88) (O'Neill and Bowie, 2007). These adaptors recruit downstream signaling molecules, forming an extensive signaling platform, also called the Myddosome (Motshwene et al., 2009). A coordinated series of phosphorylation and ubiquitination events, involving the E3 ubiquitin ligase TRAF6, ultimately causes the release of cytosolic NF-κB from its inhibitor, IκBα (Wang et al., 2001). NF-κB then translocates into the nucleus to induce transcription of pro-inflammatory cytokine genes.

The production of IFN-I following TLR4 ligation is mediated via a second signaling pathway that originates from endosomes. TLR4's capacity to signal from endosomes was identified using chemical inhibitors of clathrin-mediated endocytosis, which selectively inhibited the synthesis of IFN-I, but not pro-inflammatory cytokines (Kagan et al., 2008; Tatematsu et al., 2016). These inhibitors included chlorpromazine and dynasore. Chlorpromazine is a cationic amphiphilic drug that inhibits the formation of clathrin-coated pits, the sites at which the plasma membrane invaginates (Wang et al., 1993). Dynasore instead acts on the GTPase dynamin, which regulates the scission of membrane invaginations to form early endosomes (Macia et al., 2006). Compared with cell surface TLR4 complexes, endosomal TLR4 complexes recruit a different set of adaptor proteins, comprising

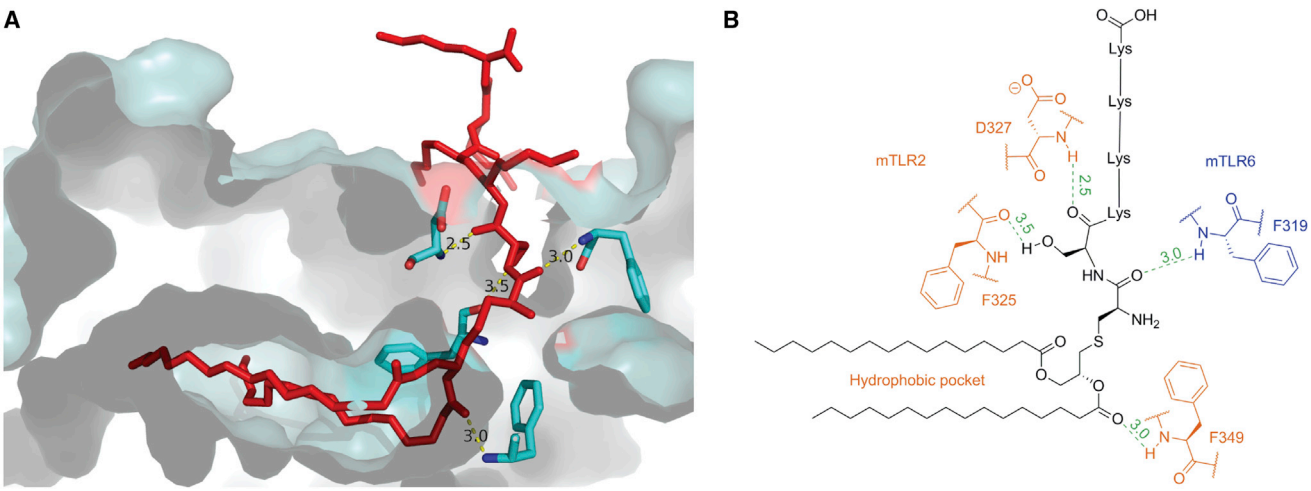

Figure 3. Ligand Binding of Pam_2CSK_4 to TLR2/6

(A) Surface rendering of the crystal structure of Pam_2CSK_4-bound TLR2/6 heterodimers (Kang et al., 2009). Key interacting residues are highlighted.

(B) Residues interacting with Pam_2CSK_4. Depicted in orange are TLR2 residues, depicted in blue a TLR6 residue. Green dashed lines represent potential hydrogen bonds with calculated distances (in Å). The four C-terminal lysine residues of the ligand have little to no interaction with the TLR2/6 complex. The two palmitoyl tails fit inside a hydrophobic pocket present on TLR2.

the TRIF-related adaptor molecule (TRAM) and the TIR domain-containing adaptor-inducing IFN-β (TRIF) (Yamamoto et al., 2003a, 2003b). TRAM and TRIF function as sorting and signaling adaptors, respectively. Subsequent recruitment of various signaling components, including TRAF3, culminates in the activation and nuclear translocation of the transcription factor IRF3. This in turn induces transcription of genes encoding IFN-I.

The spatially separated signaling pathways activating NF-κB and IRF3 are now thought to occur sequentially (Kagan et al., 2008). Switching from an NF-κB-activating pathway from the cell surface to an IRF3-activating pathway from endolysosomal vesicles is mediated through recruitment of TRAM. Prior to TLR4 stimulation, TRAM binds membrane lipids of the *trans*-Golgi network (TGN) and the plasma membrane through a bipartite N-terminal localization motif, which consists of a myristoylation motif followed by a polybasic domain (Rowe et al., 2006). Upon LPS stimulation, the TGN-localized pool of TRAM translocates to TLR4-containing endosomes (Klein et al., 2015). Here, TRAM displaces TIRAP from the TIR domain of TLR4, effectuating the switch from an NF-κB-dominated pathway to an IRF-dominated pathway (Kagan et al., 2008). It remains to be established what triggers TRAM displacement from the TGN and why TRAM does not engage TLR4 at the plasma membrane.

Translocation of the ligand-engaged TLR4 molecules from the cell surface to endosomes is not induced by the receptor itself or the downstream signaling cascade, but is instead regulated by accessory proteins, such as MD-2 and CD14. These accessory proteins were initially linked to TLR4 signaling for facilitating the transfer of LPS to TLR4 (Shimazu et al., 1999; da Silva Correia et al., 2001). Later, an additional role for CD14 in orchestrating TLR4 internalization was identified. First, CD14 targets TLR4 to lipid rafts on the cell surface, where the receptor signals through TIRAP/MyD88 (Triantafilou et al., 2002). Second, CD14 enhances endocytosis of TLR4 (Zanoni et al., 2011), resulting in a shift toward IFN-I signaling via TRAM/TRIF. The dynamics of these processes are difficult to study using standard approaches, but here "state-of-the-art" microscopes have enabled mobility studies of TLR4 and its accessory and adaptor proteins through fluorescence recovery after photo-bleaching experiments (Klein et al., 2015).

Since the signaling output of TLR4 is tightly coupled to its spatial context, general cell biological processes involved in endocytosis and protein trafficking will affect TLR signaling output. To date, a number of proteins involved in endocytosis has been shown to facilitate TLR4 internalization. These factors include p120-catenin, the GTPase ADP ribosylation factor 6 (ARF6), Annexin A2 (AxnA2), and the integrin CD11b (Yang et al., 2014; Van Acker et al., 2014; Ling et al., 2014; Zhang et al., 2015). Depletion of any of these factors abrogates LPS-induced IFN-I secretion, highlighting their importance. Yet, their exact mechanisms of action remain to be resolved. AxnA2 and p120 reduce TLR4 surface levels, thereby skewing TLR4 signaling away from the MyD88-driven production of pro-inflammatory cytokines (e.g., TNF-α, IL-6, IL-1β) in LPS-treated murine macrophages (Yang et al., 2014; Zhang et al., 2015). This indicates that the process of TLR4 internalization acts as a double-edged sword: it both dampens the pro-inflammatory response and enhances IFN-I secretion.

The concept that a surface-expressed TLR is internalized into endosomes from which it induces signals leading to IFN-I secretion also applies to TLR2 (Figure 4A). TLR2 was long considered the archetypical cell surface receptor for inducing potent pro-inflammatory responses upon sensing acetylated lipoproteins. For this reason, small-molecule TLR2 ligands were rapidly developed to be used as pro-inflammatory stimulants in vaccines, as described above. The discovery that TLR2 induced IFN-β production in murine inflammatory monocytes upon stimulation with inactivated murine cytomegalovirus and vaccinia virus (Barbalat et al., 2009) shed new light on additional effector functions of this receptor. Internalization of TLR2 was essential for IFN-I production, but not for the secretion of pro-inflammatory cytokines. This was concluded from experiments in which endocytosis or

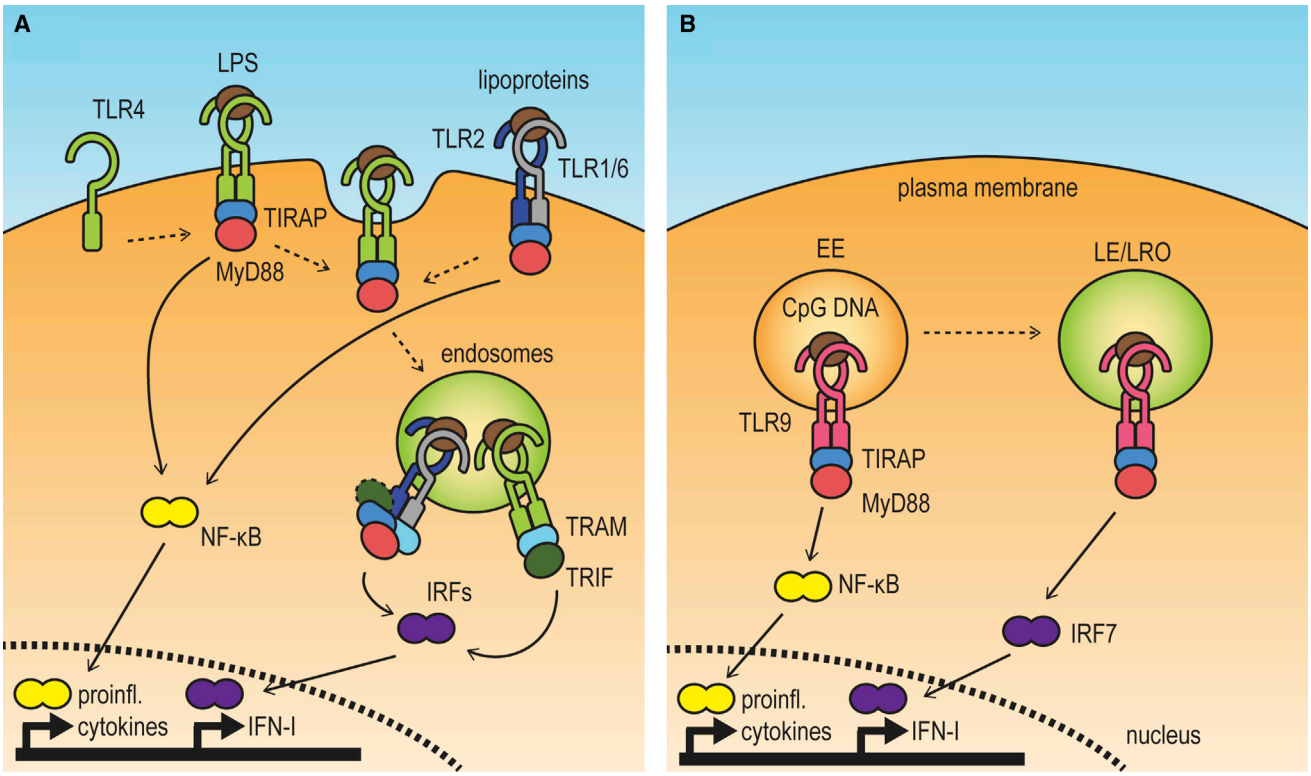

Figure 4. TLRs Induce Distinct Signaling Cascades Depending on Their (sub)Cellular Localization

(A) At the cell surface, ligand-bound TLR4 dimers assemble a signaling complex by engaging TIRAP and MyD88. This induces a signaling cascade that results in the production of pro-inflammatory cytokines via NF-κB. Internalized TLR4 dimers are retained in endosomes, where they trigger signal transduction via TRAM and TRIF, leading to the activation of IRF3 and induction of IFN-I. Although less well described, there are indications that TLR2/1 and TLR2/6 heterodimers can also induce the activation of NF-κB and IRFs from the cell surface and endosomes, respectively.

(B) The signaling cascades induced by TLR9 to activate NF-κB or IRF7 both involve TIRAP and MyD88, but originate from distinct endosomal populations. From early endosomes (EE), TLR9 induces NF-κB activation, resulting in the production of pro-inflammatory cytokines. In contrast, TLR9 activation from late endosomes (LE) and/or lysosome-related organelles (LRO) results in IRF7-dependent production of IFN-I.

Dashed lines represent trafficking pathways; solid lines represent signaling pathways.

endosomal maturation were inhibited by cytochalasin D and chloroquine, respectively. Intriguingly, IFN-I was not induced in inflammatory monocytes with low doses of synthetic TLR2 ligands. In a later report, IFN-I production by bone marrow-derived macrophages (BMDMs) was observed at higher concentrations of synthetic TLR2 ligands (Dietrich et al., 2010). An IFN-I signaling pathway was induced, both by stimuli that mimic lipoproteins (Pam_2CSK_4, Pam_3CSK_4, and FSL-1), and by the glycolipid agonist lipoteichoic acid. The capacity of TLR2 to induce IFN-I therefore does not appear to rely on the nature of the ligand by which it is activated and is likely operational downstream of TLR2/1 and TLR2/6 heterodimer complexes. To prove this, knockout studies would be required, as most cells express TLR1 and TLR6. Since the initial observation of TLR2-induced IFN-I production, there has been a growing body of evidence for a model where TLR2, like TLR4, assembles NF-κB-activating signaling platforms at the plasma membrane and IRF-activating platforms at endosomal vesicles.

In contrast to TLR4, bifurcation of TLR2 signaling events has only been known for a few years. Therefore, little is known yet about the factors that regulate the spatiotemporal aspects of TLR2 signaling. So far, studies have primarily focused on identifying which adaptor proteins are recruited by TLR2 at the plasma membrane and at vesicles of the endolysosomal system. It is worth mentioning that these studies have mainly been performed using mouse cells, and a recent report suggests certain mechanistic aspects of TLR2 signaling may vary between mice and humans (Brandt et al., 2013). The signaling platform assembled by TLR2 at the cell surface resembles that of TLR4. Both receptors employ the adaptor proteins TIRAP and MyD88 to induce signals that activate NF-κB via TRAF6, leading to the production of pro-inflammatory cytokines (Yamamoto et al., 2002). Of note, TIRAP is dispensable at high doses of TLR2 ligand (Kenny et al., 2009), which possibly allows sufficient amounts of MyD88 to couple to TLR2 in the absence of TIRAP.

In endosomal vesicles, TLR2 and TLR4 employ different sets of adaptors. In addition, TLR2 appears to affect a broader range of genes than TLR4, with IRF3 and also IRF7 activated upon stimulation (Barbalat et al., 2009). Unlike TLR4, the IFN-I inducing signaling platform assembled by TLR2 critically depends on MyD88 (Barbalat et al., 2009; Dietrich et al., 2010). TRAM is part of endosomal TLR2 signaling platforms, interacting both with TLR2 and MyD88 (Stack et al., 2014). Its importance for signal transduction follows from cells expressing a TRAM mutant incapable of associating with endosomes and immortalized TRAM-deficient BMDMs: both cell types are thwarted in IFN-I production

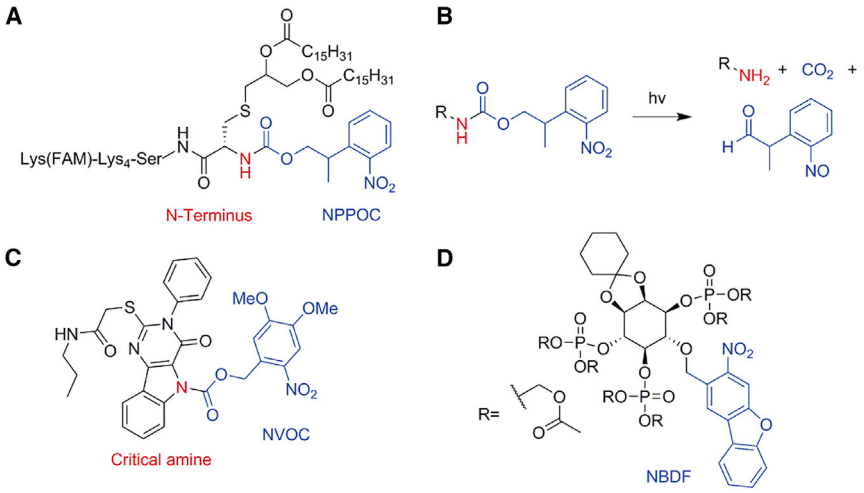

Figure 5. Photocaged Ligands and Uncaging by Photoirradiation
(A) The TLR2 ligand Pam_2CSK_4K(FAM) protected with NPPOC (blue) at the N terminus (red).
(B) A typical uncaging reaction of an *ortho*-nitrobenzyl-derivative initiated by photoirradiation.
(C) A pyrimido[5,4-b]-indole-based TLR4 ligand protected with NVOC (blue) on the amine that is critical for its activity (red).
(D) The two-photon excitation compatible group NBDF (blue) can be applied to cage agonists such as IP3.

following TLR2 stimulation (Stack et al., 2014; Nilsen et al., 2015). Whether TRIF, the signaling adaptor recruited by TRAM in case of TLR4 stimulation, is also required for signaling by endosomal TLR2 is more controversial. Nilsen et al. (2015) observed a partially impaired induction of CCL5, CCL4, and IFN-β by TLR2-stimulated TRIF-deficient BMDMs. Overexpressed fluorescently labeled TLR2 and TRIF co-localized at endosomal vesicles in HEK293 cells (Nilsen et al., 2015). In contrast, Stack et al. (2014) did not detect a TRIF-dependence in the output of IFN-I by BMDMs. The precise involvement of TRIF regarding endosomal TLR2 signaling, therefore, remains to be established.

In summary, both TLR4 and TLR2 assemble distinct signaling platforms at the plasma membrane and endosomal vesicles, which in turn induce the activation of NF-κB and members of the IRF family, respectively. Numerous factors involved in protein trafficking and accessory proteins of TLR4 have been identified that control the translocation of TLR4 and its adaptors to endosomal vesicles. Controlled translocation of TLR4 proved to be critical for shaping the induced innate response. It is likely that similar processes regulate TLR2-induced responses, which will need to be elucidated in future research. Potential candidates include accessory molecules for TLR2 ligand binding, such as CD36, CD14, and mannose binding lectin (Hoebe et al., 2005; Manukyan et al., 2005). The biological implications of inducing distinct signaling occurring from different cellular locations are not yet fully known. Perhaps, this might have evolved to fine-tune the innate immune response depending on the pathogen. The kinetics of the microbe-induced trafficking of the TLRs would allow the cell to induce a balanced response to the pathogen (Tan and Kagan, 2016). This would explain how the promiscuous receptor TLR2 can elicit tailored responses to a wide variety of natural ligands.

Knowledge of the dynamics by which TLR2 and TLR4 are regulated will aid in unraveling the spatiotemporal complexity of signaling by these receptors. This can only be investigated to a certain extent with classical synthetic TLR ligands because they are limited to bulk activation of cells. Next, we discuss new chemical tools that allow conditional control of TLR activation, which could prove very useful to study TLR signaling dynamics within individual cells.

Photocaged TLR2 and TLR4 Ligands as Tools to Study Signaling Dynamics

A new class of reagents that may aid in elucidating the spatiotemporal complexity of TLR signaling dynamics are photocaged TLR ligands. Photocaging (the protection of ligands to a non-binding state using photolabile protecting groups) has been performed on, among others, TLR2 and TLR4 ligands, yielding tools by which TLR activation can be conditionally controlled using light.

The aforementioned crystal structure (Figure 3A) and SAR studies of the TLR2 ligand Pam_2CSK_4 (**2**) indicated that a substitution at the N terminus of the cysteine renders the ligand inactive (Wu et al., 2010b). Using this information, a photocaged TLR2 ligand was developed (Mancini et al., 2015). The caging strategy here relies on preventing the hydrogen bond between F319 and the amide bond of the construct to be formed (Figure 3B). This may be facilitated by pure steric hindrance of the protecting group present on the cysteine amine. However, the protecting group used is also a hydrophobic moiety capable of interacting with the hydrophobic wall, potentially preventing the LRR11 loop shift from occurring. This can lead to an absence of proximity of F319, thus negating the hydrogen bond formation. Using this rationale, a 2-(2-nitrophenyl)propyloxycarbonyl (NPPOC) group was introduced on the N terminus, indeed rendering the ligand inactive (Figure 5A). NPPOC is an *ortho*-nitrobenzyl (NB)-derived photocleavable group, which undergoes UV-mediated intramolecular hydrogen atom transfer onto the nitro-group leading to its elimination upon irradiation with UV light (Woll et al., 2007) (Figure 5B). To be able to visualize the photocaged ligand, the fluorophore carboxyfluorescein (FAM) was incorporated. FAM was attached to the terminal lysine residue, because the lysine residues of Pam_2CSK_4 do not interact with TLR2 or TLR6 (Prass et al., 1987). Incubation of TLR2-expressing cells with the photocaged TLR2 ligand resulted in a pre-existing pool of ligand-bound TLR2 molecules, without inducing their activation. The ligand was distributed on the plasma membrane and in endosomes, consistent with TLR2 trafficking. Subsequent UV irradiation induced nuclear translocation of p65, a subunit of NF-κB, in the fibroblast cell line 3t3 and release of TNF-α from BMDMs indicating TLR2 activation.

In the case of TLR4, activation can be controlled using a photocaged ligand based on the synthetic agonist pyrimido[5,4-b]-indole. SAR studies indicated that substitutions on the indole

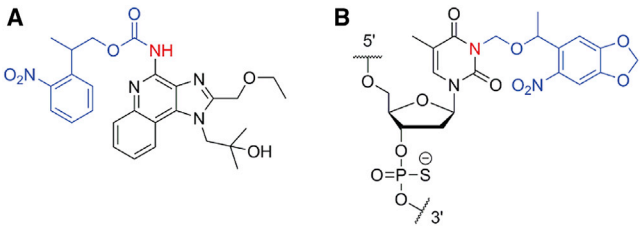

Figure 6. Ligands for NA-Sensing TLRs Protected with Photocages
(A) The TLR7 and TLR8 dual agonist resiquimod protected with NPPOC (blue) on the amine that is critical for its activity (red).
(B) A photocaged thymidine residue residing in a phosphorothioate-linked CpG oligodeoxynucleotide. The photocage nitropiperonyloxymethyl (blue) on the nitrogen (indicated in red) prevents TLR9 activation.

nitrogen are not tolerated (Chan et al., 2013). A photocaged ligand was synthesized by alkylating the critical indole nitrogen with a nitroveratryloxycarbonyl (NVOC)-group (Stutts and Esser-Kahn, 2015) (Figure 5C). Like NPPOC, NVOC is also cleaved upon UV irradiation. Using UV/Vis spectroscopy and high-performance liquid chromatography analysis, conversion into the free ligand following UV irradiation was detected. The caged TLR4 agonist was shown to induce p65 nuclear translocation and NF-κB-dependent transcriptional activity in cells only after UV exposure. A first proof-of-principle for exerting spatial control over immune activation was delivered with the caged TLR4 ligand. Restricting UV irradiation, using a pinhole with an area of 3.1 mm^2, induced p65 nuclear translocation only in cells within the pinhole area. Currently, UV-mediated photoexcitation of TLR4-expressing cells has not been achieved with higher spatial resolution. Further reduction of the area of photoactivation toward the order of sub-micrometer resolution would allow biologists to extract information from activating TLRs at specific subcellular compartments, which can lead to the induction of distinct signaling pathways as discussed above. This level of spatial control cannot be achieved by UV excitation alone, since photons above and below the focal point, as well as scattered photons outside of the focus beam, have the capacity to uncage ligands.

One way to enable the assessment of TLR signaling from specific (sub)cellular sites, without further reducing the volume of excitation, is to spatially restrict the photocaged ligands to the specific site of interest. This has successfully been applied in a chimeric receptor model, in which the erythropoietin (Epo) binding domain of the Epo receptor has been fused to the TIR-signaling domain of TLR4 (Duc and Huse, 2015). A photocaged Epo mimetic peptide (NVOC-EMP1) was immobilized on culture dishes via a streptavidin-biotin linkage. This enabled activation of the chimeric receptor specifically at the plasma membrane upon UV irradiation. In this model stimulation of single cells, as visualized by nuclear translocation of p65, was obtained using a mosaic digital diaphragm system that allows decaging in a circle with a diameter of about 5 μm. To our knowledge this approach of restricting ligands has not yet been applied to TLR-specific ligands.

A different approach to obtain a higher level of spatial control is the use of photocaging groups that are removable by two-photon excitation (TPE). During TPE, two photons of infrared (IR) light carrying roughly half the energy necessary to excite an electron from its ground state to its excited state are simultaneously absorbed (Palikaras and Tavernarakis, 2001). Two different IR lasers are positioned perpendicular to the sample, generating a focal point with a high radiation intensity. TPE quadratically depends on light intensity, therefore IR photons out of the focal point have almost no chance of exciting a photocaging group. Using this technique, the volume of excitation can be reduced to 1 fL (Klán et al., 2013), roughly 2% of the volume of a vesicle with a diameter of 1 μm. The prospect of uncaging ligands within single, small, TLR-carrying vesicles is intriguing and may add to the chemical toolbox available to study spatiotemporal effects. An additional advantage of TPE is that the use of less energetic IR photons reduces the potential for inducing cytotoxic events, such as inducing DNA damage or the generation of reactive oxygen species.

The photolytic efficiency of most NB derivatives used for one-photon excitation is too low for TPE applications. The photolytic efficiency can be enhanced by increasing the size of the conjugated system, the planarity of the cage, and the amount of strong donor/acceptor couples (Bort et al., 2013). Such adjustments are often paired with an increase in hydrophobicity, an unwanted property for experiments performed under aqueous conditions. This hurdle has held back the development of groups applicable for TPE in biological experiments. To fill this gap, Momotake et al. (2006) synthesized the nitrodibenzofuran (NDBF) group as an alternative caging moiety with a high photolytic efficiency. The NDBF group photolyzes 16–160 times faster than other NB derivatives and is currently one of the most efficient NB-derived photocages, competed with only by a recent improved version of this photocage (Komori et al., 2016). The NBDF group has already successfully been applied as a cage for iron (Kennedy et al., 2010), nucleosides (Lusic et al., 2010), and inositol-1,4,5-triphosphate (IP3) (Figure 5D) (Kantevari et al., 2012). These combined results suggest that the use of two-photon uncaging approaches could be an exciting prospect in conjunction with caged TLR ligands to study the dynamics of TLR signaling from specific (sub)cellular sites.

The Dynamics of Intracellular NA-Sensing TLRs

The NA-sensing TLRs, TLR3, TLR7, TLR8, and TLR9, signal from intracellular vesicles along the endolysosomal pathway. Despite this seemingly simple spatial organization, recent discoveries indicate that TLR9, and possibly also TLR7 and TLR8, induce signaling pathways that activate NF-κB and IRF7 from functionally distinct intracellular vesicles (Figure 4B).

The first evidence that NA-sensing TLRs induce distinct signals depending on spatial context was obtained using two structurally diverse synthetic TLR9 ligands: CpG-A and CpG-B oligodeoxynucleotides (ODNs). While mouse pDCs produce IFN-I in response to multimeric CpG-A, monomeric CpG-B only induces the release of pro-inflammatory cytokines (Honda et al., 2005). The difference in cytokine output was proposed to rely on the distinct intracellular trafficking properties of these ligands. CpG-A was retained in early endosomes for a prolonged time, while CpG-B rapidly translocated to late endosomes and lysosomes. A slow progression of CpG-A through the endolysosomal pathway is specific for pDCs. In cDCs, the trafficking properties of CpG-A resemble those of CpG-B, and both ligands induce pro-inflammatory cytokines. While not normally released

by cDCs, IFN-I are produced in response to CpG-A complexed with the liposomal transfection agent DOTAP. This reagent enhances endosomal retention of CpG-A.

Recently, an endosomal cargo trafficking pathway has been identified as a critical regulator for inducing IFN-I expression downstream of TLR9. Upon TLR9 stimulation in pDCs, the cargo shuttling protein adaptor protein-3 (AP-3) mediated translocation of TLR9 from early endosomes to lysosome-related organelles (LROs), and this was a prerequisite for the activation of IRF7 (Sasai et al., 2010). Recruitment of AP-3 to TLR9-containing vesicles depended on PIKfyve, an enzyme controlling phosphoinositide (PI) metabolism (Shisheva, 2008). PI(3)P is found on early endosomes and PIKfyve catalyzes its phosphorylation to PI(3,5)P2, which is abundantly present on late endosomes (Catimel et al., 2008). Inhibition of PIKfyve impaired the translocation of TLR9 and CpG-A into LROs (Hayashi et al., 2015). In addition, the production of IFN-I downstream of TLR9 was specifically blocked by inhibiting PIKfyve in murine bone marrow-derived DCs (BMDCs).

These combined data support a model in which TLR9 traffics between functionally different endosome populations to induce distinct signaling cascades. Remarkably, TLR7, TLR8, and TLR9 induce distinct signaling cascades to activate NF-κB and IRF7 using the same adaptor set consisting of TIRAP and MyD88 (Bonham et al., 2014). The mechanism by which different pathways can be induced using the same adaptors remains incompletely understood. The observation that TLR9 drives IFN-I production from LROs suggests that cellular factors, facilitating the assembly of a signaling platform capable of activating IRF7, may be restricted to these vesicles. TLR3 is different from other NA-sensing TLRs in relying on TRIF, instead of TIRAP and MyD88, for the activation of both NF-κB and IRFs (Brubaker et al., 2015). Whether TLR3-induced signaling pathways also depend on spatial context awaits further evaluation.

Caging Strategies for NA-Sensing TLRs

Recent efforts to conditionally control the activation of NA-sensing TLRs using UV light as an external trigger encompass the development of photocaged ligands for TLR7, TLR8, and TLR9. Based on the crystallographic data mentioned above, the C4 amine of the dual TLR7 and TLR8 agonist resiquimod was suggested to be critical for its activity (Zhang et al., 2016b). Thus, an NPPOC group was introduced at this position, rendering the ligand inactive (Ryu et al., 2014) (Figure 6A). A mouse macrophage cell line with an NF-κB reporter (RAW-Blue) was used to confirm that the product of UV-irradiated NPPOC-resiquimod induced TLR7 and TLR8 activation. In addition, BMDCs treated with the photocaged ligands secreted the cytokines IL-6, IL-12, and TNF-α only after UV irradiation. Another imidazoquinoline, the TLR7-specific agonist imiquimod, was successfully caged in a similar manner.

For TLR9, several CpG ODNs were synthesized carrying the photocage nitropiperonyloxymethyl, by incorporating pre-protected thymidine phosphoramidite building blocks (Govan et al., 2015) (Figure 6B). These thymidine residues were either equally distributed over the ODN or concentrated at both termini. Both caging strategies prevented activation of TLR9 in the Burkitt's lymphoma cell line Namalwa, yet a more pronounced photoactivation for the ODN with terminally protected thymidine residues was observed by measuring IL-6 secretion. The authors hypothesized that the central section of this ligand may be able to bind TLR9 without inducing activation, analogous to the above-discussed photocaged TLR2 ligand. Whether caged CpG ODN is able to create a pre-existing pool of agonist-bound TLR9 awaits further investigation.

Currently, one of the major limitations of using light-activated caging reagents is the level of spatial precision that can be achieved. As discussed above, two-photon uncaging approaches may further reduce the volume of excitation, allowing TLR ligands to be activated within single specific endosomes. Another recently emerged field of chemistry, which might be of great use here, exploits bio-orthogonal chemical uncaging strategies (Li and Chen, 2016). Chemical cages, such as azides, can be removed using a Staudinger reduction reaction and have previously been used to cage peptide epitopes (Pawlak et al., 2015) and catalytic sites in enzymes (Luo et al., 2016). In terms of uncaging rates and yields, the most promising chemical cages are *trans*-cyclooctene cages (Fan et al., 2016). These were used initially to allow triggered drug release from antibody-drug conjugations (Versteegen et al., 2013; Rossin et al., 2016), but have also been used to cage enzyme activities (Li et al., 2014; Zhang et al., 2016a). Although none of these chemical caging strategies have been applied on TLR ligands yet, they may prove to yield useful tools for the localized activation of TLRs by directing chemical uncaging agents to specific (sub)cellular sites.

Outlook

The previous static model of TLR signaling has undergone major revisions in recent years. It has become evident that TLR signaling is regulated dynamically within cells, where the specific subcellular localization and timing of TLR activation affect signaling outcome. New reagents, techniques, and chemistries are appearing fast, allowing spatial and temporal control over TLR activation. These chemicals are expected to become valuable tools for delineating the complex dynamic nature of TLR signaling. Controlled activation of TLRs is essential for potent antimicrobial immune responses. At the same time, undesired TLR signaling may induce deleterious immune responses in the case of misregulation or overactivation toward infection, or autoimmunity toward self-agonists. A better understanding of the dynamic cellular processes that regulate TLR signaling will guide the rational design of novel therapeutics to effectively prevent these instances of immune pathogenesis.

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