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Space and Time: New Considerations About the Relationship Between Toll-like Receptors (TLRs) and Type I Interferons (IFNs)

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Abstract

The Toll Like Receptors (TLRs) and the type I Interferons have critical roles to play in innate immunity. In this review we will discuss new developments relating to the important area of TLR/IFN cross regulation

Keywords

TLR; Interferon; IFN- β ; IFNAR; macrophages; TLR4; TLR2; LPS; Vaccinia virus; TRAF3; CD14; TRAM; TRIF; IRF3; IRF7

I. Introduction

Within the field of innate immunity, type I interferons (IFNs) and the Toll-like receptors (TLRs) have long been recognized as two central pillars governing responses to infectious microbes. These two critical elements of the innate response do not exist in isolation, but rather, exhibit multiple levels of cross-talk that is essential for the full efficacy of each system. This review will detail and comment on several areas of TLR-IFN counter-regulation that are highly significant within contemporary research. In particular, we hope to highlight an ongoing evolution in the model that describes how members of the TLR family relate to the signaling pathways that govern induction of type I IFN and vice versa. The longstanding traditional model was largely static and based on the identity of the receptor. In this schema, individual TLRs were grouped based on their ability to directly induce IFN secretion in response to a purified ligand traditionally assayed in an innate immune cell such as a macrophage. This static understanding is being revised by new work we will discuss that shows the importance of place, *i.e.*, where in a cell and/or on which type of cell a TLR is located, as well as time, *i.e.*, when we are looking during a sequence of signaling events, when discussing a TLR's role in IFN induction.

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The TLRs: Biology and Signaling to Type I IFNs

The TLRs are a family of type I transmembrane pattern recognition receptors (PRRs) that have the capacity to recognize a broad array of widely distinct microbes, ranging from bacteria to viruses, fungi, and parasites. In mammals, the TLR family presently consists of 13 members. TLRs 1–9 are conserved between humans and mice with TLR11, TLR12, and TLR13 being functionally lost in humans. Members of the TLR family share a common structural organization that is the basis of their identification as TLRs. The structural TLR prototype is composed of an N-terminal ectodomain, a single type I transmembrane domain, and an intracellular Toll/interleukin (IL)-1R Resistance (TIR) domain. The N-terminal ectodomain is typified by the presence of leucine-rich repeat sequences (LRRs) that mediate recognition by TLRs of their respective ligands. The TLR TIR region allows for interactions among TLRs and adapter proteins, and initiates signal transduction cascades [1].

The individual ligands recognized by TLRs vary in their structure and chemistries. Microbial ligands (referred to as “pathogen or microbial-associated molecular patterns (PAMPs or MAMPs) identified to date include: triacylated and diacylated lipopeptides, respectively (TLR 1/2 and 2/6 heterodimers) [2], extracellular double-stranded RNA (TLR3) [3], lipopolysaccharide and many others (TLR4) [2], bacterial flagellin (TLR5) [4], single-stranded extracellular RNA as well as select small molecules (TLR7/8) [5, 6], CpG DNA (TLR9) [7]. TLR4 is the only TLR that does not directly bind ligand, but rather, is dependent upon a non-covalently associated co-receptor called MD2 to bind ligand and initiate dimerization of the TLR4 molecules.

Ligation of TLR3 or TLR4 leads to direct induction of the IFN- β promoter. TLR3 is associated with endosomes and TLR4 becomes internalized into endosomes after LPS stimulation. TRAM and TRIF are a pair of TLR-recruited adaptor proteins [8, 9]. Ligation and dimerization of TLR4 leads to association with TRAM and subsequent recruitment of TRIF [10]. TRIF, in turn, nucleates a signaling complex comprised of the ubiquitin ligase TRAF3 and the TANK-binding kinase 1 (TBK-1) [11, 12]. Activated TBK-1 phosphorylates and activates the transcription factor IRF3. Once activated, IRF3 primarily serves to drive robust transcriptional activation of the type I IFN- β response, as well as induction of other IRF-3-dependent genes [13]. IFN- β can also be induced through the MyD88 adaptor following ligation of endosomally located TLRs 7, 8, and 9 [14]. In such cases, MyD88 recruitment to the TIR domain mediates direct binding and auto-activation of the transcription factor IRF7. Once activated, IRF7 (as with IRF3) translocates to the nucleus where it drives expression from the IFN- β promoter [15, 16].

Type I IFNs

The type I IFNs are a family of ubiquitously expressed pleiotropic cytokines that utilize a common type I IFN receptor (IFNAR). In human, the type I IFN family consists of a single IFN- β gene and 13 IFN- α genes. The large number of IFN- α genes are thought to have arisen through gene duplication and display some cell type specificity of expression. The type I IFNs are potently and rapidly induced by select TLRs and, once induced, are secreted and act on a broad array of tissue types. Downstream of the IFNAR, IFNs induce hundreds of genes through the hierarchical action of the JAK-STAT proteins. In brief, binding of IFNs

to IFNAR changes the conformation of the intracellular portion of this heterodimeric receptor allowing auto- and trans-phosphorylation of kinases tyk2 and JAK1. These events permit association of the transcription factors STAT1 and STAT2 with the IFNAR that allows the phosphorylation and activation of these transcription factors [17]. Following activation, the STAT proteins enter the nucleus and drive transcription of hundreds of IFN-responsive genes. Many of the induced genes are directly antimicrobial and, in particular, anti-viral. However, many of the IFN-induced genes modulate other aspects of the innate immune response including TLR signaling and function [18]. As well as governing the innate response, IFNs serve as an essential point of contact between the innate and adaptive responses

II. Regulation of IFN induction through TLR4 localization

In vivo induction of type I IFN by TLR4 through the above described TRIF-dependent pathway has been known for a significant period of time to be a critical source of IFN during microbial infection *in vivo*. The molecular requirements for TLR4 to engage MyD88 and/or TRIF adaptors were initially thought to be identical [8–10]. However early work from our lab and that of others revealed that there may, in fact, be distinct interactions required for TLR4 to induce MyD88- and TRIF-dependent genes [19]. CD14 is a, high affinity GPI-anchored, surface-expressed or soluble co-receptor for TLR4 that is required at low doses of LPS for optimal induction of all MyD88-dependent genes. However, the requirement for CD14 can be overcome with increasing concentrations of LPS [19]. This data suggested that CD14 plays predominantly a kinetic role by facilitating transfer of LPS to the TLR4/MD2 complex. However, while high levels of LPS could restore induction of MyD88-dependent genes, induction of IP-10 (CXCL10), now known to be TRIF-dependent, could not be restored by high doses of LPS. Thus, there appeared at first glance to be a subset of LPS-responsive genes for which CD14 was an absolute, rather than, a kinetic requirement.

The next breakthrough in our understanding of distinct TLR4 signaling pathways was revealed when a mutant mouse strain, given the name ‘heedless,’ was identified. Macrophages from this strain were capable of inducing the classic complement of inflammatory cytokines in response to the lipid A, a region of LPS, but were incapable of inducing IFN- β . Genetic mapping of the heedless mutation revealed that it lay within the coding region of CD14 and resulted in the truncation of 83 C-terminal amino acids [20]. Furthermore, heedless mutants could be rescued by complementation with wild-type CD14, thereby linking CD14 to a unique molecular relationship with the induction of type I IFN. However, the precise molecular and intermolecular determinants remained obscure.

The next piece in the molecular puzzle arrived with work carried out Kagan et al. who showed that TLR4 activates MyD88 and TRIF pathway signaling, not simultaneously from the cell surface as had been previously thought, but rather, the MyD88 and TRIF pathways display spatio-temporally distinct cell biology [21]. In this model, TLR4 first engages MyD88 at the cell surface and initiates MyD88-dependent signaling from this location. TLR4 is then internalized, and enters into the intracellular compartment of Rab5-positive early endosomes where it is competent to bind the TRAM and TRIF adaptors and to initiate signaling leading to the induction of type I IFNs. Importantly, this work also demonstrated

that the subcellular localization of TRAF3 was critical for determining the capacity of TLRs to induce type I IFN. If TRAF3 were directed to the inner cell membrane through fusion with a lipid-binding domain, it conferred upon TLR2 the capacity to induce IFN- β where TLR2 normally would not.

A major link in this conceptual chain was also contributed by Zanoni et al. In this report, it was finally shown that CD14 expression is the key and essential determinant of whether TLR will be endocytosed and initiate TRIF-dependent type I IFN induction in response to LPS [22]. In response to LPS, expression of CD14 allows for a Syk-dependent signal transduction cascade to be activated that is required for TLR4 endocytosis. Chemical inhibition of Syk or PLC γ 2 activities abrogated TLR4 endocytosis as well as IFN- β induction. Interestingly, the requirement for CD14 could be bypassed in dendritic cells exposed to “phagocytic cargo,” such as whole *E. coli* organisms. TLR4 could then be internalized in the absence of CD14. This phagocytic bypass was, however, not observed in macrophages and precisely how this phenomenon occurs in dendritic cells is unclear.

Despite all of the work that has been done, key questions remain in the spatiotemporal regulation of type I IFN induction. Among the most significant questions is how precisely CD14 is able to contribute to the activation of the Syk and PLC γ signal transduction cascades. This is a significant question as CD14 is GPI anchored to the cell surface or found in soluble form and does not contain an intracellular portion of the receptor. It has been speculated that CD14 governs the organization or composition of cell surface lipid rafts and that by doing so, it facilitates select signal transduction.

Recent work by our group has indicated that there may also be ligand specificity with respect to the requirement for CD14 in the induction of TLR4-dependent IFN induction (submitted). This may be biologically significant given that these studies have identified relevant non-LPS ligands for TLR4 that may be able to induce type I IFN in a CD14-independent fashion. More work remains to be done to elucidate this.

TLR2: A Type I IFN-inducing TLR?

In the early days of TLR biology, a key finding was that while LPS, a TLR4 ligand, could readily induce IFN- β , that in turn, acted in an autocrine/paracrine fashion to induce STAT1 phosphorylation in murine macrophages, known ligands for TLR2, including the synthetic triacylated lipopeptide, Pam3Cys, did not [23]. The basis for this discrimination between IFN-inducing and non-inducing TLRs was later shown to be determined by differential adaptor usage. IFN-inducing TLRs, including TLR4, utilized the adapter TRIF to signal to TBK-1 and IRF3, while other TLRs including TLR2 and 5 were restricted to using MyD88-dependent signaling [9]. Based on this paradigm, and the long held belief that type I IFNs are anti-viral in nature, it was long assumed that TLR2 was predominantly a sensor for bacterial lipoproteins. Recent findings in several areas of investigation have challenged this model. It has now been shown that, in fact, multiple viruses representing significant human pathogens encode TLR2 ligands among their structural proteins. Viruses in this group include Varicella-zoster, Cytomegalovirus and respiratory syncytial virus have all been shown to either directly activate TLR2 or to persist for greater duration in a TLR2-deficient host [24–26]. In the absence of direct IFN induction, how TLR2 contributes to antiviral

defense was not clear. Recent results in our laboratory exploring mechanisms of TLR cross-talk may provide a potential clue to this. We and others have shown that murine macrophages exposed to synthetic ligands for TLR2 broadly suppressed production of classic pro-inflammatory cytokines such as IL-6 and IL-12 in response to subsequent stimulation with TLR4 ligand. This result had been coined as TLR-induced “cross-tolerance,” and was thought to be a protective response to prevent hyper-inflammation in cases of disseminated infection as occurs during sepsis [27]. Unexpectedly, prior exposure of macrophages to TLR2 ligands not only failed to suppress subsequent TLR4-driven IFN- β induction, but also increased subsequent IFN production by as much as 10-fold [28]. Analysis of TLR4 signal transduction following TLR2 priming revealed that IRF3 activation was vastly increased for a given dose of challenge ligand, while NF- κ B activation was maintained and the activation of other transcription factors, such as AP-1, was markedly inhibited in the primed cells. It was further shown that following TLR2 stimulation, macrophages were rendered resistant to infection by a number of RNA viruses including VSV and influenza [28]. While such an observation could not be explained easily by regulation of TLR4 signaling, this engendered an exploration of the effects of TLR2 priming on the biology of other virus sensing PRRs, *i.e.*, the RIG-Like Receptors (RLRs). Exposure to TLR2 ligands were found to vastly increase production of type I IFNs in response to a viral surrogate, synthetic poly I:C, introduced into macrophages by transfection. Introduction of poly I:C by transfection resulted in a dramatic increase in the activation of transcription factor IRF3. An investigation of the molecular mechanism revealed that TLR2 priming of macrophages resulted in a marked up-regulation of the K63 ubiquitin ligase TRAF3, while having no impact on the expression of the closely related family member TRAF6. TRAF3 is uniquely positioned at a common node in the type I IFN-inducing pathways downstream of both the TLRs and the RLRs, thus making it ideal to regulate both systems. TRAF3 levels are not similarly regulated by ligation of other MyD88-utilizing TLRs, such as TLR5 [28]. Thus, this type of cross-talk between signaling families of receptors may have significant consequences when patients are co-infected with Gram positive bacterial pathogens, such as *Streptococcus pneumoniae*, and RNA viruses, such as influenza. Within such a co-infection context, IFN- β may be predominant in the cytokine milieu and have differential impacts on the bacterial and viral infections.

TLR2 as a cell type-specific inducer of type I interferon

While almost all of the work examining the capacity of various differential adaptor-utilizing TLR subsets has produced a keen understanding of this area of innate immunology, an unfortunate limitation is that almost all of this work was done in a very limited set of innate cells, *i.e.*, macrophages, occasionally dendritic cells, and sometimes, mouse embryo fibroblasts (MEFs). What we are learning is that the responses of different cell types may bias our understanding of whether a given TLR can induce type I IFN. New work with TLR2 stimulation has raised the possibility of tissue specificity for the capacity to induce type I IFN. TLR2 stimulation by UV-inactivated Vaccinia Virus can induce IFN- β ; however, this only occurs in inflammatory monocytes [29]. One of the most significant findings to come out of this work is the observation that even in inflammatory monocytes, synthetic bacterial lipopeptides, such as Pam3Cys, do not induce type I IFN. This opens up the possibility for an entirely new level of discrimination between ligands from different classes

of organisms and that this capacity for ligand discrimination might determine the downstream outcome of gene induction.

TLR2: Novel adaptor interactions

The longstanding paradigm for TLR adaptor usage has held that TLR2 ligands do not engage the TRIF adaptor pathway and that cytokine induction by TLR2 is unimpaired in TRIF KO macrophages [9]. However, recent reports challenge these assumptions in the case of specific cytokines, in particular CCL5. CCL5 (also known as RANTES) is potently induced by all TLR2 ligands. Lien and colleagues have demonstrated that the TLR2-dependent induction of CCL5 is significantly dependent on TRIF-mediated activation of the transcription factor IRF3 [30]. This TLR2-dependent TRIF pathway requires endosomal internalization of TLR2 that is mechanistically strongly reminiscent of the manner in which TLR4 has been shown to spatially restrict its interaction with TRAM and TRIF. Important elements of these functional interactions between TLR2 and the TRIF pathway remain to be elucidated, including whether TLR2 can engage TRIF in all cell types.

A second recent study by Stack et al. demonstrates direct IFN- β induction by TLR2 ligands in immortalized murine bone marrow-derived macrophages (iBMDM) [31]. In these experiments, stimulation of TLR2 by Pam3Cys or MALP-2 induces IFN in a process involving both the adaptors MyD88 and TRAM, but not TRIF. This TRAM-dependent IFN induction requires endocytosis of the receptor, and results in the activation of IRF7 rather than IRF3. The TLR2 dependent endosomal activation of IRF7 is postulated to be analogous to the endosomal MyD88-dependent activation of IRF7 through TLR9.

Beyond TLRs: effects of IFN on TLR-Induced Macrophage Functional Polarization

Recently, there has been an explosion of interest in defining the role and molecular mechanisms of macrophage functional polarization. Broadly defined, macrophage polarization reflects a continuum between the classically activated (M1) differentiation state on one end, achieved through stimulation of macrophages with LPS and/or IFN- γ , to the Alternatively Activated (M2) state on the other end of the spectrum, loosely defined by the up-regulation of a constellation of M2-specific “markers” *e.g.*, arginase-1, mannose receptor, etc.; (reviewed in [32]). M1 macrophages are typified by the expression of high amounts of pro-inflammatory mediators such as IL-1 β , IL-6, and TNF α , as well as by the potent capacity to phagocytose and kill microbes. M2 macrophages, on the other hand, produce relatively low levels of classical inflammatory cytokines and are typified instead by the production of anti-inflammatory cytokines, as well as IL-4 and/or IL-13. Functionally, the M2 macrophage population is induced during parasitic worm infections and/or situations of wound repair. Interestingly, the M1 and M2 polarization states appear to be plastic, with the degree and type of polarization reversible with an alteration in cytokine milieu or microbial stimulation [33]. In fact, in macrophages, a transition from the M1 to M2 states may be a natural process of resolution of the inflammatory phase. Our lab first observed that type I IFN may have an important role to play in the transition from the M1 to the M2 state in certain infections. Respiratory Syncytial Virus (RSV) induces early M1 polarization, followed by a transition to the M2 state both *in vitro* in infected peritoneal macrophages, as well as *in vivo* in the lungs of infected mice and cotton rats [34]. The *in vivo* transition from

M1 to M2 following RSV infection appears to be a protective response, as animals genetically deficient in the shared component of the IL-4/IL-13 receptor (IL-4R $\alpha^{-/-}$), that is required for development of the M2 differentiation state, display markedly greater lung pathology following RSV infection. Importantly, the RSV-induced transition of lung macrophages to the M2 state also requires the expression of TLR4. Interestingly, this TLR4-induced M2 transition is potentially influenced by the expression of type I IFN. Intranasal RSV infection of mice deficient in expression of IFN- β (IFN- $\beta^{-/-}$) results in prolonged M1 activation of BAL macrophages and resulted in an almost complete ablation of RSV-induced expression of canonical M2 markers such as arginase-1, Ym1, and FIZZ1 [34]. How on a molecular level Type I IFN is influencing the transition to the M2 differentiation state remains to be elucidated, but may involve antagonistic interplay of STAT transcription factors known to be important in the M1 or M2 fate determination [35].

Concluding Remarks

It is clear that our understanding of the TLRs as inducers of the type I IFN response needs to evolve in order to incorporate the new dynamics of TLR cell biology with respect to sub-cellular trafficking, cell type restriction, and temporally regulated co-receptor interactions. Many of the most exciting results in this area have come to light within the last five years, and it is doubtless that the pace of discovery will only quicken. The TLRs are no longer ‘on/off’ switches, but finely tuned situational governors of type I IFN induction. Much remains to be done however, including elucidating how a given TLR may differentially regulate IFN responses to different pathogens with distinct ligand chemistries. In addition, little has been done to investigate what impact the type I IFNs, once induced, may be having to alter and regulate the function of the TLRs themselves. Hopefully these answers will be forthcoming.

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