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The role of endosomal toll-like receptors in bacterial recognition

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Abstract. – BACKGROUND: Infections caused by extracellular Gram positive bacteria are still a major health problems. Better understanding of the mechanisms underlying immune responses to these organisms is key to develop pharmacological agents, including vaccines, to control these infections.

OBJECTIVE AND PERSPECTIVES: The objective of this review is to highlight the importance of nucleic acid-sensing, intracellular Toll-like receptors in innate immune recognition and in host defenses against extracellular bacteria.

CONCLUSIONS: Toll-like receptors 7 and 9 have a major role in inducing host-protective type I interferon responses in conventional dendritic cells in response to streptococci and other extracellular gram positive bacteria. Moreover an as yet unidentified MyD88-dependent receptor is likely responsible for proinflammatory cytokine induction in response to these pathogens.

Key Words:
Pattern recognition receptors, Interferon, Nucleic acids, Endosomes.

Introduction

The ability of multicellular organisms to preserve their integrity and to control growth of smaller organisms colonizing their surfaces depends on their ability to recognize all kinds of microbes or, in other words, to detect the entire universe of "microbial not self"[1]. With the possible exception of erythrocytes, all cells of the body are equipped with a wide variety of receptors, encoded in the germline, which are able to sense the presence of a selected set of evolutionary-conserved microbial substances. Such receptors are particularly abundant in cells of the innate immunity system, which have the function to patrol body sites and to provoke protective responses, including inflammation and adaptative immunity responses (i.e. antibody production and T-cell activation). Current pharmacological research is heavily focusing on molecules capable of stimulating, inhibiting or otherwise modulating the activity of these innate immunity receptors. Practical applications include, for example, the development of agonists to be used as adjuvants (i.e. of molecules capable of augmenting vaccine responses[2-4]) and antagonists for the treatment of chronic "autoinflammatory" and autoimmune diseases[5].

Molecules capable of sensing the presence of microbial signatures include Toll-like receptors (TLRs), retinoid acid-inducible gene (RIG)-like receptors (RLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs)[6–8]. These sensors can be membrane associated (such as TLRs) and reside on cell surfaces or be strategically located in intracellular vacuoles, such as endolysosomes, in order to sample vacuolar contents. RLRs and NLRs, instead, reside in the cytosol. The present review will mainly focus on the function of intracellular or endolysosomal Toll-like receptors, which have evolved to sense the presence of microbial nucleic acids after they are released from microorganisms internalized by host cells using endocytosis. Because they are capable of recognizing nucleic acids, which are life's most essential molecules, endosomal TLR are potentially able to sense any kind of infectious agents we may ever encounter, including viruses, fungi, bacteria, and protozoa. Despite this, there is a strong feeling among immunologists that virus detection is the true *raison d'etre* for intracellular TLRs. No doubt, some viruses, such as the herpes simplex virus[9] may be detected predominantly by these receptors. Since, however, the importance of intracellular TLRs in infections by non-viral pathogens tends to be underestimated, we will summarize here some evi-

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dence indicating that these TLRs are absolutely required for cytokine responses to extracellular bacteria (particularly Gram positive bacteria). First, we will concisely review some essential features of the biology of intracellular TLRs.

Signal Transduction by Endosomal TLRs

TLR family members, including the endolysosomal TLRs, are type I membrane proteins composed of a ligand-binding ectodomain containing tandem copies of leucine-rich repeats (LRRs), a single-pass transmembrane domain, and a conserved cytoplasmic Toll/interleukin-1 receptor (TIR) domain used for transducing signals to the cytosol. Ligand-induced TLR dimerization leads to binding of signaling adaptor molecules of which the most important are myeloid differentiation factor 88 (MyD88), which binds TLRs 7, 8, and 9[10] and TRIF (TIR domain- containing adaptor inducing interferon [IFN-β]) which bind TLR3. These adaptors mediate the recruitment of a series of kinases and ubiquitin ligases, which lead to the formation of specific macromolecular signaling platforms. One of these platforms, termed Myddosome, created by interactions between MyD88 and the kinases IRAK4 and IRAK2/1 has been well defined[11,12]. The TLR signaling cascade ultimately results in the activation and nuclear translocation of several transcription factors, including nuclear factor kB (NF-kB), adaptor protein 1 (AP-1), IFN-regulatory factor 3 (IRF3), IRF1 and IRF7, which together initiate the expression of genes encoding type I IFNs and other cytokines, chemokines, chemokine receptors, and costimulatory molecules.

Endolysosomal TLRs include TLR3, recognizing double-stranded RNA (dsRNA), TLRs 7 and 8 specific for single-stranded RNA (ssRNA), and TLR9 specific for DNA[13,14]. These sensors are expressed in several cell types of the immune system, including conventional dendritic cells (cDCs), plasmacytoid DCs (pDCs), macrophages, and B cells. Expression of endolysosomal TLRs differs widely among these cell types. These differences, together with differential intracellular TLR localization, deeply influence the type of pathogens recognized and the responses elicited. Major histocompatibility complex (MHC) class II molecules are apparently also required for optimal TLR signaling[15] by a mechanism taking place in endosomes and involving interaction of MHC class II molecules, through CD40, with the Bruton's tyrosine kinase, which then interacts with MyD88 and TRIF[16].

Spatial Regulation of Endosolysosomal TLR Activation

Intracellular TLR accessibility to ligands as well as quality and quantity of the response is strictly regulated by: (1) trafficking of internalized (endocytosed) material; (2) trafficking of TLR themselves[17]. Intracellular TLRs are perfectly capable of being activated by self nucleic acids. Such activation does not occur under normal circumstances by virtue of mechanisms preventing the presence of self nucleic acids in the specific endosomal compartments where intracellular TLR activation is allowed to occur. Indeed, as it will be outlined below, intracellular TLRs, can be functional only in acidified endosomes containing various proteases[18]. Under normal circumstances, host nucleic acids are sequestered in the nucleus, in the cytosol or in mitochondria, and the small amounts that might escape extracellularly are digested by extracellular DNases and RNases. In other words, physical separation of host nucleic acids from their intracellular receptors is the single most important factor of self/not-self discrimination.

In resting cells, TLRs 3, 7, 8, and 9 are localized in several intracellular compartments. They predominantly reside in the endoplasmic reticulum (ER), but also -to a lesser degree- in endosomes and lysosomes. Upon cell activation (caused not only by nucleic acids, but also by other stimuli, such as lipopolysaccharide) endosomal TLRs are quickly translocated from the ER to the endosomal compartment[19].

Several ER-associated proteins have been shown to act as chaperones and to mediate such translocation. Unc93b1 resides in the ER and is required for trafficking of TLRs 3, 7, and 9 to endolysosomes. The 3d missense mutation of Unc93b1 causes lack of signaling by TLRs 3, 7, and 9 and susceptibility of mutant mice to infection by various pathogens[20]. Other genes are apparently involved in trafficking of TLRs to the cell surface and to endosomes. One example is gene GP96 (an ER paralog of the HSP90 family). In fact, macrophages deficient in this molecule do not respond to TLRs 1, 2, 4, 5, 7, or 9 ligands[21,22]. Trafficking of TLRs 1, 2, and 4 from the ER to the plasma membrane (and of TLRs 7 and 9, but not TLR3, to the endolysosomes) is also affected by PRAT4A[23]. More recently, AP-3, a 4-subunit (d, b3A, m3A, s3), clathrin-associated adaptor protein complex, was involved in TLR7 or TLR9 activation. In particular, DCs from AP-3-deficient mice showed defective production of

type I IFNs in response to TLR7 or TLR9 agonists, but NF-kB-mediated induction of IL-12p40 was unaffected[24]. Moreover AP-3 was required for trafficking of TLR9 and Unc93b1 to late, but not to early endosomes. Therefore, it appears that intracellular TLRs, together with Unc93b1, might first be transferred by an AP-3-independent mechanism to early endosomes, resulting in NF-kB-transactivation, and then to late endosomes by an AP-3-dependent mechanism, thus resulting in IRF7 activation and production IFN-beta production. Similarly, the viperin protein is required for TLR7- and TLR9-mediated production of type I IFNs by pDCs[25], but not for the production of other inflammatory cytokines.

A crucial, unique requirement for endosomal TLR activation is receptor processing by means of acidic proteases. The ability of intracellular TLRs to activate downstream signal transduction pathways, but not their ability to bind ligands, is acquired only in endolysosomes on cleavage of a major portion of their ectodomain by cathepsins[26]. Cathepsins are indeed required for activation of all endosomal TLRs in all examined cell types[27].

Overall, there appear to be a number of mechanisms (including the above described formation of late endosome-specific activation platforms and receptor cleavage by resident acidic proteases) ensuring that functional, nucleic acid-specific TLRs are confined to endolysosomes, thereby precluding their engagement by self-nucleic acids and ensuring that responses are initiated under conditions leading the release of nucleic acids from pathogens.

Intracellular TLRs and IFN Responses to Bacteria

A first hint, as to the role of these receptors in responses to extracellular bacterial pathogens, came from studies dealing with type I IFN production. It was well known that exposure to bacterial products induced type I IFN[1], but the functional consequences of such responses on the outcome of bacterial infections were incompletely understood. While the crucial role of type I IFN in host protection against viral infections has been universally recognized for more than 5 decades, these cytokines had been traditionally assigned a minor role in antibacterial host defenses. Surprisingly, it was found that IFN-αβ signaling was crucial also in host defenses against extracellular gram positive and gram negative bacteria[28]. These effects could be related to the ability of IFN-β to boost macrophage production of proinflammatory cytokines and bactericidal nitrogen radicals in the presence of bacterial stimulation. Lipopolysaccharide was not involved in these effects, which were also observed using Gram positive bacteria. At the same time, IFN-β was also suspected to play a role in hyperinflammatory sindromes associated with viral, as well as non-viral infections[29-34].

These findings prompted studies aimed at identifying the receptors and signal transduction pathways involved in type I IFN production after stimulation with Gram positive bacteria. It was found that cDC (conventional dendritic cells), but not pDC (plasmacytoid dendritic cells) or macrophages, can produce high quantities of IFN-β following bacterial degradation in phagolysosomes, and that such responses required a novel recognition pathway involving TLR7, the adaptor MyD88 and the transcription factor IRF1 (interferon regulatory factor 1)[35]. These data highlighted a unifying theme in innate immunity whereby the host uses the same type of receptor (in this case, TLR7) to mount robust IFN-α/β responses against RNA viruses and bacteria, albeit in different cell types (in pDC and cDC, respectively). Moreover, these findings indicated that the endophagosomal system of cDC may be specialized to internalize and process bacteria for presentation of their nucleic acids to endosomal TLRs. In fact, it was also found that TLR9, in addition to TLR7, partecipated in activation of the novel IRF1-dependent bacterial recognition pathway, as suggested by significant reduction in IFN-β production the absence of TLR9[35]. This notion is supported by the known ability of bacterial DNA and other TLR9 agonists to induce IRF1-dependent responses in cDC[36,37]. Several considerations suggest that RNA is the bacterial ligand targeted by TLR7 in cDC. First, TLR7 is strictly RNA-specific and single-stranded RNA is its only known natural agonist[38,39]. Second, RNA is abundantly present in prokaryotic cells (usually in amounts 2 to 10 times greater than DNA), and is likely to be present in particularly high concentrations in the phagolysosomal lumen. Finally, and perhaps most importantly, the ability of GBS to induce TLR7-dependent IFN-β induction can be recapitulated by bacterial RNA and RNAse treatment greatly reduces IFN-β induction by GBS lysates. Moreover, different types of bacterial RNA had different IFN-inducing capabilities, with mRNA displaying the highest potency[35]. Collectively these data indicate that TLR7 and TLR9 appear to cooperate in bacterial recognition by recognizing bacterial RNA and DNA, respectively.

Bacterial internalization appears to be absolutely required for IFN production, in agreement with the possibility that all pathways leading to induction of this cytokine family originate from an intracellular location. As to the cell type involved in IFN responses to bacteria, it was surprising that pDCs, which can produce huge quantities of IFN after viral stimulation, are unresposive to whole bacteria[35]. However this can be simply explained by the inability of pDC to internalize bacteria, as opposed to viruses.

cDCs produced more IFN-β than did macrophages after bacterial stimulation. In macrophages, interferon responses occurred independently of the prevalent intracellular location of the stimulating pathogen and uniformly required IRF3 but not IRF1 or MyD88. In contrast, cDCs were able to use at least two distinct pathways to mount robust interferon responses to bacteria residing in cytosolic and phagosomal compartments. Responses to the former group of pathogens required IRF3, whereas responses to the latter group totally depended on IRF1 and MyD88 and partially depended on IRF7[35].

The activation of distinct sets of transcription factors by cytosolic and phagosomal pathogens may provide further insight into the mechanisms with which the innate immune system responds to organisms residing in different locations. It is likely that by avoiding progression along the phagosomal pathway, some pathogens prevent the activation of IRF1, which promotes proinflammatory and often host-protective T helper cell type 1 responses. Indeed, IRF1 but not IRF3 was required in vivo for defense against extracellular bacteria that are normally sequestered in the phagosomal pathway and are unable to escape into the cytosol[35]. In addition, TLR7 was also required for optimal defenses against extracellular bacteria. Therefore it is possible that substantial, IRF1-dependent production of type I interferon by cDCs is required for antibacterial host defenses, whereas insubstantial, IRF3-dependent production of interferon by macrophages is dispensable.

These studies emphasize a link between the infected cell type, the pathogen's intracellular location, the sensing receptors and the transcription factors involved in IFN-α/β responses[28,35]. Such a link had been previously documented during infection by viruses, which can activate IRF3-dependent responses from a cytosolic location in most cell types, and an IRF7-dependent pathway from the endosomes of pDC[1]. Apparently, parallel, cell-type specific pathways of this kind can also be activated by most bacterial pathogens, namely by those that undergo killing and degradation in cDC. Like viruses, these bacteria can induce low-grade IFN production in macrophages as well as a more robust response in a specialized DC type (e.g. in cDC in the case of bacteria and pDC in the case of viruses) through the activation of endosomal TLRs.

The mechanisms underlying the unique ability of cDC to mount robust anti-bacterial IFN responses are presently unclear. It is possible that endophagosomal trafficking in these cells, which entails efficient phagocytosis and rapid translocation of ingested material to late, mature compartments, may facilitate the processing of prokaryotic organisms and subsequent presentation of their nucleic acids to endosomal TLRs. It is reasonable to assume that only lysosomal-type vacuoles are sufficiently "degradative" to allow digestion of the thick and hardy prokaryotic envelope and subsequent exposure of bacterial nucleic acids. Accordingly, TLR7-dependent recognition was triggered at a distal point along the phagosomal maturation pathway[35] (e.g. after phagolysosome fusion). The mechanism described here for IFN induction by bacteria in cDC is in sharp contrast with TLR9-dependent IFN-α induction by DNA oligonucleotides in pDC, which is initiated at the in early, but not late endosomes[17,40] (i.e. under conditions that are associated with the uncoating of most viral genomes).

Intracellular TLRs and Proinflammatory Responses to Non-Viral Pathogens

Indications concerning the potential role of an as yet unidentified endosomal TLR in recognition of gram positive bacteria came from studies with Group B Streptococcus (GBS) an important neonatal pathogen. In accordance with a crucial role of the TLR system in the recognition of GBS, MyD88 is essential for the induction of pro-inflammatoty cytokines (e.g. proIL-1β, IL-12p40 or TNF-α) in macrophages when they are exposed to these bacteria[41]. However, the specific TLR, or combination of TLRs, which interacts with GBS particles on one side and MyD88 (myeloid differentiation primary response gene 88) on the other has not been identified in macrophages. Although lipoproteins are not essential in the context of stimulation by bacterial cells, extracellularly released lipoproteins do stimulate pro-inflammatory cytokine production through TLR2/6 activation[42].

There are some indirect indications, using various kinds of RNases on whole heat-killed bacterial cells gram-positive bacterial cells, that

macrophages detect the presence of prokariotyc-type single-stranded RNA by a mechanism involving an unknown UNC-93B- and MyD88-dependent receptor, which is different from classical nucleotide-sensing TLRs[6,43]. Similarly, in cDCs, the release of either IL-1β or TNF-α is entirely MyD88-dependent, but is not affected by the absence of other TLR adaptors, such as MAL, TRIF, and TRAM. Moreover, IL-1β release was not affected by lack of TLR2, TLR4, TLR7 and TLR9[6]. These data indicate that, similar to TNF-α, pro–IL-1β is induced by a MyD88-dependent mechanism that, nevertheless, does not require any of the better-characterized TLRs, such as TLR2, TLR4, TLR7, and TLR9. The requirement for UNC93b, a chaperone protein responsible for TLR traslocation to the endosomal compartment, would suggest the involvement of an as yet unidentified intracellular TLR. Clearly, further studies are needed to identify the MyD88- and UNC-93B-dependent recognition receptor responsible for pro–IL-1β induction and TNF-α secretion after stimulation with whole Gram-positive bacteria. It is remarkable, however, that both pro-inflammatory cytokine and type I IFN responses to these bacteria require phagocytosis, phagosomal acidification and receptor translocation to the endosomal compartment[6] (unpublished data).

The complete dependence on Myd88 and on phagosomal pathways for recognition of gram positive extracellular bacteria is in sharp contrast with mechanisms involved in fungal recognition. These involve at least two different cellular mechanisms associated with the production of two different sets of defense factors. The first mechanism, underlying the production of TNFα, relies predominantly on the detection of cell-wall structures, mostly insoluble glucan, by receptors located on the host cell surface, such as dectin-1 and other C-type lectins[44,45]. This mechanism does not necessarily require phagocytosis and is largely independent from TLRs or TRL adaptors. The second mechanism depends on progression of the organisms along the classical phagolysosomal route and apparently on the release of nucleic acids from the cell envelope. DNA and RNA are then detected by, respectively, TLR9 and TLR7 and trigger MyD88- and IRF1-dependent responses leading to the production of IL-12p70, NO and IFN-β[44,45]. TLR7-9 and IRF1-dependent production of this set of mediators is apparently required for optimal anti-*Candida albicans* host defenses[45]. IRF1 deficiency resulted in more pronounced susceptibility to systemic candidosis than either TLR7 or TLR9 deficiency. Activation of the IRF1 pathway was absolutely dependent on phagocytosis and phagosomal acidification, in agreement with the known endosomal localization of TLR9 and TLR7. In contrast, TNF-α responses to whole yeast were totally independent from phagocytosis and were initiated by dectin-1, rather by TLRs. Conversely, depleted zymosan, a potent activator of the dectin-1 pathway was totally unable to induce IL-12p70 production, in agreement with the notion that depleted zymosan or other particulate β-glucan preparations are unable to stimulate IL-12p70 secretion and may actually decrease IL-12p70 induction by TLR ligands. Therefore, DCs use two completely distinct pathways to secrete primary cytokines in response to fungal recognition, only one of which requires progression through the classical phagolysosomal pathway and targets IRF1. We propose a model whereby the interaction of cell membrane receptors (e.g. dectin-1) with surface-exposed components (e.g. cell wall glucan) is sufficient to induce TNF-α in the absence of yeast internalization, whereas the induction of IL-12p70 and type I IFN requires the release of yeast nucleic acids in acidified phagosomes and their subsequent recognition by TLR7/9[44]. Our data point to the importance in terms of anti-fungal defenses of this novel phagosomal pathway and may be useful to better understand the strategies used by fungi to evade the innate immune system.

Conclusions and Future Perspectives

The cellular sites prevalently occupied by different microbes in their interaction with host cells deeply influence the ways by which the latter perceive microbial presence. Extracellular pathogens have developed means to escape phagocytosis, but after they are internalized they cannot escape progression along the endolysosomal pathway and digestion. Nucleic acid sensing TLRs, with the exception of TLR3, are important for the detection of these pathogens, likely because they are strategically located in endolysosomes. A major challenge for future research is the identification of the Myd88- and UNC93b-dependent receptor responsible for proinflammatory cytokine responses to extracellular Gram positive bacteria such as streptococci and staphylococci.

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