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Contribution of microbial-associated molecules in innate mucosal responses

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Abstract. Both innate immunity and mucosal surfaces provide the first line of defence against mucosal infections. Innate immunity is a universal and evolutionarily conserved form of host defence that senses microbial organisms. Recent advances in the field of immunology are due mainly to the discovery of the role of Toll-like receptors (TLRs), which recognize conserved microbial molecules. TLR stimulation induces specific patterns of gene expression that lead to the shaping of innate and adaptive immunity. Since mucosal tissues are colonized by innocuous microflora and challenged by infectious pathogens, activation of TLRs in epithelial and lamina propria cells must be tightly controlled to avoid inappropriate signalling that might lead to mucosal inflammation. This review aims to highlight novel insight on the molecules, pathways and gene expression networks associated with microbial recognition by TLRs and mucosal immunity.

Key words. Toll-like receptor; innate immunity; antimicrobial defence; gene expression; kinases.

Introduction

Microbial infection and local and widespread inflammation are constant risks in the mucosal surfaces of the gastrointestinal, respiratory and genitourinary tracts, which have a combined surface area of at least 400 m^2 in the adult human. These mucosal surfaces comprise the body's first line of defence against a variety of microbial organisms. Remarkably, the mucosal immune system is typically able to distinguish pathogens from both the commensal flora and innocuous dietary antigens and mount an appropriate immune response to each type of challenge [1, 2]. So it is not surprising that mucosal tissue contains the greatest number of immune cells and secondary lymphoid tissues. The mucosal immune system consists of organized mucosa-associated lymphoid tissue that is separated from mucosal surface antigens by epithelial barriers [3]. Although epithelial organization varies dramatically in different mucosal surfaces, it always provides both barrier and signalling functions to protect against infections. The intestinal mucosa is covered by a single layer of epithelial cells, and the airway lining varies from pseudostratified to simple epithelium. Multilayered squamous epithelia line the oral cavity, pharynx, oesophagus, urethra and vagina. Erosion of the epithelial barrier by inflammation, stress or disease can lead to translocation of microbial organisms into the bloodstream. If microorganisms that cross the epithelium turn out to be pathogenic, the host could die from septicaemia unless the micro-organisms are eliminated by the immune system. For this reason, the epithelial cells as well as the immune cells of the subepithelial compartment, which include lymphocytes, monocytes, macrophages, polymorphonuclear leukocytes and dendritic cells (DCs), participate in the induction of the host's innate and adaptive immune responses.

The innate immune system represents an ancient host defence system that has a great degree of specificity and the ability to discriminate between self-molecules and foreign microorganisms. Activation of the innate host defence by microbes depends on specific recognition of molecules of microbial origin, which are known as pathogen-associated molecular patterns (PAMPs) [4]. Mammalian cells detect PAMPs through pattern-recognition

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receptors (PRRs) [5]. These molecules include the members of the Toll-like receptor (TLR) family and the nucleotide-binding site/leucine-rich repeat (NBS/LRR) proteins, such as the nucleotide-binding oligomerization domain 1 (Nod1) and Nod2 [6, 7].

This review focuses on recent findings regarding the recognition of microbial molecules through the TLRs and Nods within mucosal cells and the role of these PRRs in the induction of an appropriate mucosal immune response.

Toll-like receptors

The first Toll receptor was identified in the fruit fly *Drosophila melanogaster*. *Drosophila* Toll participate in the control of infections by Gram-positive bacteria and fungi [8], in addition to its long-known function in embryonic dorsoventral patterning [9]. Numerous studies performed in the last 7 years here established that TLRs play an essential role in initiating the innate antimicrobial immune response in organisms as diverse as plants, insects and mammals [10]. Structurally, TLRs are type I integral membrane glycoproteins with molecular weights ranging from 90 to 115 kD, and belong to the superfamily of interleukin-1 receptors (IL-1Rs). TLRs and members of the IL-1R family share a conserved stretch of ~200 amino acids in their cytoplasmic region, known as the Toll/IL-1 receptor (TIR) domain. The extracellular portion of TLRs contains 19–25 contiguous copies of a motif known as leucine-rich repeat (LRR), whereas that of IL-1Rs contains three immunoglobulin domains [11]. The LRR domain consists of varying numbers of repeats, each 24–29 amino acids in length. It is thought that the LRR domains of TLRs are directly involved in the recognition of micobial components, and accumulating evidence suggests that this is probably true in the case of mammalian TLRs [11, 12], but not in the case of *Drosophila* Toll [13].

So far, 13 mammalian TLRs have been reported, 10 of which can be found in humans, designated TLR1–TLR10. In mice, the orthologue of human TLR10 is a degenerate pseudogene, but recently three more mouse TLR homologues (TLR11–TLR13) have been identified [14, 15]. Each TLR recognizes conserved microbial molecules that are consistent within a given class of microorganism. The first coupling of a ligand with a TLR was reported in 1998, with the identification of TLR4 as a receptor for lipopolysaccharide (LPS) detection [16]. The most recently identified ligand is single-stranded viral RNA that can be detected by human and mouse TLR7 and human TLR8 [17–19]. So far, a plethora of ligands has been identified for different TLRs, including lipoteichoic acid and lipoproteins for TLR2 [20, 21], double-stranded viral RNA for TLR3 [22], bacterial flagellin for TLR5 [23] and unmethylated CpG DNA common to bacterial and viral DNA for TLR9 [24]. Interestingly, some TLRs detect their ligands by forming heterodimeric complexes. To this extent, TLR2 cooperates with TLR1 or TLR6 for the detection of triacylated and diacylated lipoproteins, respectively [25–27].

TLRs are localized on the cell surface or, in the case of TLR3, TLR7, TLR8 and TLR9, in endosome-lysosome intracellular compartments of immune cells [19, 28–30]. However, their expression, localization and responsiveness can vary between different subsets of a certain cell type or be altered during cell maturation and exposure to microbes. For example, human blood monocytes express most TLRs, but their expression decreases several fold as they differentiate into immature DCs, whereas TLR3 shows exactly the opposite expression pattern [31]. TLR3 is not expressed in human or mouse plasmacytoid DCs, but again, these cells can detect viral infection since they express TLR7 (single-stranded RNA) and TLR9 (unmethylated CpG DNA) [32]. Most of our knowledge regarding the distribution and biological role of TLRs has so far been achieved by studying DCs and macrophages that have been isolated from human blood or mouse spleens, or derived from mouse bone-marrow precursors upon in vitro differentiation [32]. However, the expression pattern and role of TLRs in the antigen presenting cells that are present at the mucosal surfaces have been poorly investigated, although these cells play a pivotal role in the detection of microorganisms and the induction of mucosal immunity.

On the other hand, the contribution of TLRs in epithelial cells to mucosal immunity has just begun started to be uncovered. Intestinal and airway epithelial cells are unresponsive or hyporesponsive to endotoxins, and this is mainly because TLR4 is in low abundance, localized in the Golgi and not present on the plasma membrane of these cells [33–35]. Moreover, low expression of CD14 and MD-2, two molecules that are pivotal in the formation of a functional TLR4 complex, has also been reported in epithelial cells [36, 37]. However, infection can result in increased expression of epithelial TLR4 and localization to the apical membrane, leading to sensitization of the cells to endotoxins, as shown for human airway epithelial cells that have been previously infected with respiratory syncytial virus [35]. To date, a significant collection of human and murine epithelial cell lines and primary cells have been shown to express TLR4 [33]. Interestingly, a comparative study on the expression of TLR2 and TLR4 along the mouse gut revealed constitutive expression in mouse intestinal mucosa, but differential expression patterns along the gut segments. TLR2 and TLR4 are focalized in the colon, and following the induction of experimental colitis in mice using dextran sodium sulfate (DSS), their messenger RNA (mRNA) expression is upregulated in mucosal surfaces [34]. TLR2 is also found in airway epithelial cells and associates with the asial glycolipids

present on the apical membrane. It is believed that this interaction aids in the formation of lipid raft microdomains, which are a precondition for a functional TLR2 signalling complex [38]. Regarding TLR5 expression, it has been reported that this receptor is localized to the basolateral side of polarized T84 human colon epithelial cells. Further, it has been proposed that only bacteria that have breached the epithelial layers or have translocated bacterial products, such as flagellin, across the epithelial cells will activate epithelial TLR5 and mount an innate immune response [39]. Nevertheless, there is evidence that in Caco-2 intestinal epithelial cells, flagellin-mediated signalling can occur apically without bacterial translocation [40], suggesting that in certain epithelial cell lines or subtypes TLR5 may also be expressed apically. The use of serological expression cloning to spot commensal bacterial proteins that could contribute to the pathogenesis of inflammatory bowel disease (IBD) identified flagellins as a class of immunodominant antigens that stimulate pathogenic intestinal immune reactions both in mice and in humans [41]. Moreover, TLR5 is expressed in lung epithelial cells, and a common stop codon polymorphism in the TLR5 ligand-binding domain is associated with human susceptibility to pneumonia caused by the flagellated bacterium *Legionella pneumophila* [42]. Finally, the newly identified TLR11 is expressed in the epithelial cells of the kidney and bladder, and upon intraurethral infection with the uropathogenic bacterium *Escherichia coli* 8NU, the kidneys, but not the bladders, of TLR11-deficient mice are massively infected compared with normal mice [14]. Although the natural bacterial ligand for mouse TLR11 is still unknown, these data suggest that TLR11 is important in preventing infection of internal organs of the urinary system.

TLR signalling cascades

Upon ligation, TLRs signal intracellularly via their cytoplasmic TIR domains to promote the expression of genes involved in immune activation [6, 43]. Studies have been conducted to decipher how the activation of different TLRs that detect a plethora of diverse microorganisms, such as bacteria, viruses and fungi, lead to different patterns of gene expression [6, 43, 44]. Originally, all TLRs were thought to signal only through the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88). Although this is still true for TLR5, TLR7 and TLR9, we now know that part of the specificities of TLRs can be attributed to the existence of different adaptor molecules. So far, four TLR adaptor proteins have been identified and characterized: MyD88; TIR-domain-containing adaptor protein (TIRAP), also known as MyD88-adaptor-like protein (MAL); TIR-domain-containing adaptor inducing interferon β (IFN-β) or TRIF, also known as TIR-domain-containing adaptor molecule 1 (TICAM-1); and TRIF-related adaptor molecule (TRAM), also known as TIR-domain-containing adaptor molecule 2 (TICAM-2) [6, 43]. Most of our knowledge regarding which adaptor molecules are downstream of each TLR comes from the analysis of deficient mice that have been generated by gene targeting or identified by forward genetics [6, 43]. In summary, TIRAP is essential for MyD88 signalling through TLR2 and TLR4 and leads to nuclear factor kappaB (NF-κB) activation [45, 46], TRIF functions in the MyD88-independent pathway to induce IFN-β and is downstream of TLR3 and TLR4 [47, 48], and TRAM is involved in the activation of the MyD88-independent/TRIF-dependent signalling pathway through TLR4 and leads to the expression of IFN-inducible genes (fig. 1) [49]. There is one more TIR adaptor protein, the sterile alpha and HEAT/Armadillo motifs (SARM), which is an orthologue of a *Caenorhabditis elegans* TIR-domain-containing protein, but its function in TLR signalling remains unclear [50].

Following ligand engagement, the TLRs/IL-1Rs dimerize and undergo the conformational changes required for the recruitment of downstream signalling molecules. TLR3 or TLR4 signalling leads to the phosphorylation of the transcription factor IFN regulatory factor 3 (IRF3). Phosphorylated IRF3 translocates to the nucleus and activates the production of type I IFN-β, which subsequently induces the expression of IRF7 and further production of IFN-α and IFN-β. In the case of the MyD88-dependent pathway, the adaptor molecule MyD88 binds to the cytoplasmic portion of the TLR through interaction between the individual TIR domains. Then, IL-1R-associated kinase (IRAK)-4, IRAK-1 and tumor necrosis factor (TNF)

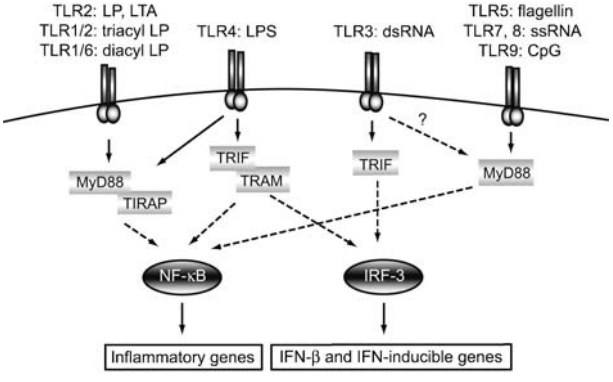

Figure 1. TLRs and their adaptors. The adaptor molecule MyD88 associates with the TIR domain of TLRs and transduces signals that induce NF-κB activation, which leads to the production of inflammatory genes. The MyD88-dependent pathway downstream of TLR2 and TLR4 also requires TIRAP. TRIF exists downstream of TLR3 and TLR4, mediates the MyD88-independent pathways and leads to the induction of IFN-β and IFN-inducible genes. TRAM is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway. LP, lipoprotein; LTA, lipoteichoid acid; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA.

receptor-associated factor 6 (TRAF6) are recruited to the receptor complex, where the association of IRAK-1 with MyD88 via their respective death domains takes place. IRAK-4 then phosphorylates IRAK-1, and the phosphorylated IRAK-1, together with TRAF6, dissociates from the complex and associates with another complex that is composed from transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and TAB3. The complex of TRAF6, TAK1, TAB1 and TAB2 further forms a larger complex with Ubc13 and Uev1A, which induces activation of TAK1. Activated TAK1 phosphorylates the inhibitor of the NF-κB (IκB)-kinase (IKK) complex, which consists of IKKα, IKKβ and NF-κB essential modulator (NEMO)/IKKγ, and thereby induces activation of the NF-κB-dependent transcription. In addition, TAK1 activates mitogen-activated protein (MAP) and stress-associated protein (SAP) kinases, such as extracellular signal-related kinase (ERK), p38 and JUN N-terminal kinase (JNK). Activation of NF-κB leads to the production of proinflammatory molecules such as TNF-α, interleukins (IL-1, IL-6 and IL-12), chemokines, and cyclooxygenase-2 (COX-2). A more detailed description of the molecular mechanisms that mediate TLR signaling can be found in the reviews by Akira and Takeda [6], and Beutler [43].

Intracellular recognition by Nods

More recently, the cytoplasmic NBS/LRR proteins have been identified as molecules that play a key role in host defence [51]. These proteins are characterized by the presence of three distinct domains: a C-terminal LRR domain, a central NBS domain or NACHT (neuronal apoptosis protein, CIITA, HET-E and TP1) and an N-terminus protein-protein interaction domain organized in either CARD (caspase-activating and recruitment domain), PYD (pyrin domain) or BIR (baculovirus inhibitor of apoptosis protein repeat). The NBS/LRR proteins belong to the CATERPILLER family (CARD, transcription enhancer, R(purine)-binding, pyrin, lots of leucine repeats) [7]. Based on the functional features and phylogenetic analysis of the NACHT alignment, the CATERPILLER family is divided in four subfamilies: Nod CIITA, ICE-protease activating factor (IPAF) and NALP [7]. To date, the members of the Nod subfamily have been the most extensively analysed. Nod1 (CARD4) and Nod2 (CARD15) were identified as proteins with structural homology of their CARD domains with that of the apoptosis regulator APAF-1, an activator of caspase 9 [7]. Both Nod1 and Nod2 sense bacterial peptidoglycan (PGN); however, each detects distinct PGN molecular motifs. Nod1 recognizes a unique diaminopimelate-containing N-acetylglucosamine, N-acetylmuramic acid tripeptide muropeptide [52] found mainly in Gram-negative bacterial PGN and the dipeptide γ-D-glutamyl-meso-d aminopimelic acid [53]. On the other hand, Nod2 mediates responsiveness to the muramyl dipeptide MurNAc-L-Ala-D-isoGln (MDP), which is conserved in PGNs of both Gram-negative and Gram-positive bacteria [54, 55]. Nod1 expression is ubiquitous in human adult tissues, whereas Nod2 expression is restricted mainly to leukocytes, DCs and epithelial cells [56, 57]. Also, Nod1 expression is upregulated by IFN-γ in intestinal epithelial cells, and Nod2 expression is increased in vitro by various inflammatory stimuli such as LPS, TNF-α or IFN-γ [57, 58]. Moreover, *Streptococcus pneumoniae* infection induces upregulation of Nod1 and Nod2 expression both in bronchial epithelial cells and in mouse lung tissues [59]. Interestingly, mutations in the gene encoding Nod2 were shown to be associated with Crohn's disease, a chronic intestinal inflammatory disease known to be influenced by both genetic and environmental factors [60, 61]. Although the mechanisms by which Nod2 increase susceptibility to Crohn's disease are unclear, these findings emphasize the key connection between bacteria and innate immune recognition as the basis of this disease. Moreover, point mutations in the NACHT domain of Nod2 were shown to segregate with Blau syndrome, a rare autosomal dominant disorder characterized by early-onset granulomatous arthritis, uveitis and skin rash with camptodactyly [62].

Upon stimulation, Nod1 and Nod2 proteins physically interact with a common downstream signalling molecule, the Rip2 kinase (also known as RICK or CARDIAK), through homophilic CARD-CARD interactions. Rip2 is essential for NF-κB by both Nod1 and Nod2, since activation of NF-κB by Nod1/Nod2 expression is completely abolished in Rip2-deficient fibroblasts [63]. A recent study using dominant negative overexpression and small interfering RNA (siRNA) experiments have shown that in addition to Rip2, the signal-transducing molecules IRAK, IRAK2, TRAF6, NIK, TAB2 and TAK1 are involved in NF-κB activation by Nod2 proteins in pneumococci-stimulated cells [59]. Thus, Nod-mediated signalling may be partially homologous to TLR/MyD88 downstream signalling [6].

TLRs above and below the barrier

Mucosal immunity relies on a series of bidirectional epithelial barrier-immune cell interactions to block the penetration of pathogenic microbes while preserving tolerance to commensal flora and particulate antigens. The mucus layer, the secretion of immunoglobulin A (IgA) and the production of antibacterial defensins from the epithelium provide the first physical barrier between colonizing microbes and the mucosa [64]. The differential expression and/or localization of different PRRs in epithelial compartments may provide a mechanism of discrimination between 'harmful' and 'beneficial' mi-

crobes passing through the mucus to reach the epithelial barrier. However, soluble proteins and bacteria do cross the epithelial barrier and have to be confined in the mucosa by its immune system [65]. An extensive collection of innate tissues underline the epithelial barrier and includes professional inflammatory effectors and antigen-presenting cells (DCs and macrophages) localized in distinct anatomical compartments [66]. In the intestinal lamina propria, a diffuse network of macrophages monitors epithelial barrier permeability. It is anticipated that TLR engagement in these cells may induce a wide spectrum of antimicrobial activities, including phagocytosis and the production of biocidal reactive oxygen/nitric oxide radicals as well as numerous cytokines and chemokines with proinflammatory and anti-inflammatory activities [44, 67]. Challenge studies have indicated that commensal bacteria can be cleared within hours by macrophages [68]. Failure of these phagocytic biocidal mechanisms renders the mucosa intolerant to the intestinal flora, with severe consequences [69]. Furthermore, mucosal DCs can meet numerous TLR agonists either indirectly, through cross-presentation from microfold (M) cells on the dome of Peyer's patches [66], or directly, by extending dendrite-like processes through epithelial tight junctions to sample the luminal microbes [65, 70]. Interestingly, DCs primed with commensal bacteria are restricted to the mucosal immune system and do not circulate in the periphery, suggesting that the immune system becomes ignorant of these microbes and/or tolerant to associated antigens [65, 66]. TLRs may modulate several DC functions in the mucosa that are central to the establishment of effector lymphocyte responses, such as antigen uptake, migration, activation and co-stimulation, as well as T helper 1 (Th-1) or Th-2 polarization [71]. However, a definitive connection between differential TLR engagement on mucosal DCs with mucosal ignorance and tolerance remains to be determined.

Integration of TLR and cytokine signals in mucosal homeostasis

The continuous presence of bacteria in the immediate proximity of mucosal PRRs indicates that PRR signals may be under precise control to avoid overt stimulation. In this respect, it appears that TLRs have direct ways of modulating their signaling capacity, aside from their differential expression, through the direct induction of negative-feedback mechanisms. For example, IRAK-M is a kinase-deficient IRAK molecule expressed in monocytes and macrophages. In contrast to other IRAKs that are ubiquitously expressed, IRAK-M is induced following TLR engagement [72]; its temporal intracellular increase interferes with the release of IRAK1 from the MyD88/IRAK4 complex and thus prohibits subsequent interaction of IRAK1 with TRAF6. Similarly, an alternatively spliced short isoform of MyD88 (MyD88s) that lacks the IRAK4 binding domain is induced in monocytes following TLR4 engagement to compete the interaction of MyD88 with the receptor [73]. Finally, the extracellular molecule SIGIRR is expressed in monocytes following LPS stimulation but does not bind to TLR ligands; instead, it interacts transiently with either TLR receptors or signalling components through its TIR domain to reduce the threshold of TLR signalling [74]. It is conceivable that such direct negative-feedback mechanisms may also apply to other mucosal effector cells (e.g. epithelia and DCs) through as yet unidentified interactions.

Besides these direct mechanisms of feedback modulation, TLR signals are combined with cytokine networks that are responsible for mucosal homeostasis. Animal models of IBD have indicated that the production of pleiotropic cytokines such as TNF-α, IL-1, IL-6, IL-10, IL-12, IL-18 and others must be precisely regulated in the mucosal microenvironment to avoid the risk of widespread inflammation [75]. Aberrant cytokine production, immune deviation and epithelial damage are processes known to support the development of IBD, presumably through the induction of a response to commensal flora that were previously tolerated [75, 76]. Furthermore, IBD-related *NOD2* mutations correlate with the uncontrolled production of such cytokines [77]. It is anticipated that PRR-induced immunomodulatory cytokines feed back in an autocrine/paracrine fashion to interfere with TLR signaling cascades through their own receptors in order to preserve intestinal homeostasis.

The interplay between cytokine and TLR signalling can be directly inferred by the shared use of selected intracellular mediators. For example, both TLRs and IL-1Rs share the use of IRAKs as central adaptor kinases to transduce their signals [44]. Similarly, TRAF6 is activated both by members of the TLR/IL-1R family and by members of the TNF receptor family [78]. Although definitive evidence is currently lacking, the differential recruitment of these adaptors by TLRs or cytokine receptors may directly modify their corresponding thresholds of activation. Such interference mechanisms can also include the differential activation of downstream kinases. For example, TAK-1 is essential in mediating the activation of NF-κB and MAPKs/SAPKs by TLRs [79, 80]. However, TAK1 associates TAB1, TAB2 and TAB3 in a differential fashion to modulate various TLR and cytokine signals [79, 80]. Similarly, the central MAPKKK, MEKK3 can be activated either indirectly or directly by TLR and cytokine signals, following association with TRAF6 [81].

In addition to the direct negative feedback and cross-interference mechanisms, PRR signalling can be inhibited by anti-inflammatory mediators in the mucosa. Several animal models of IBD have indicated that intestinal homeostasis may be perturbed due to the absence of anti-in-

flammatory controls [75]. The most characteristic example of cytokine-mediated homeostasis in the intestinal mucosa is the interplay between TNF-α and IL-10. These cytokines can be concomitantly produced in response to infectious stimuli, whereas their receptors are widely distributed in mucosal compartments including epithelia, neutrophils, macrophages, DCs and lymphocytes [82–85].

In the majority of IBD models, TNF-α appears as a common pathogenic denominator despite the variability of the instigating stimuli or the genetic defects leading to the development of IBD [86]. Clinical trials have shown that anti-TNF-α antibodies provide marked clinical benefits in human Crohn's disease patients [87]. The biosynthesis of TNF-α in innate cells is quite complex and includes the activation of numerous signalling modules, such as IKKs that modulate its transcription, the Tpl2/Cot kinase that modulates the nucleocytoplasmic export of TNF-α mRNA and the p38 and JNK kinases that modulate TNF-α mRNA stability and translation [88–92].

Conversely, IL-10 has emerged as a potent anti-inflammatory and immunomodulatory cytokine for the mucosa [83]. The LPS-induced biosynthesis of IL-10 has been poorly studied but seems to rely heavily on transcriptional mechanisms through SP1 and signal transducer and activator of transcription 3 (STAT3), which may be direct or indirect targets of LPS-stimulated pathways [83]. Mice deficient in IL-10, IL-10 receptor or associated signalling molecules develop IBD [75, 76]. In addition, aberrations in TNF-α production due to the absence of IL-10 function are one of the causative effects for the development of IBD [85]. The interplay between TNF-α and IL-10 reciprocates the interplay between the TLR signals that induce TNF-α production and the Janus kinase (JAK)/STAT/suppressors of cytokine signalling (SOCS) pathway elicited by IL-10R. SOCS proteins were originally identified as negative-feedback inhibitors of interleukin and interferon signalling by directly inhibiting the functions of JAK/STAT [93]. The IL-10/TNF-α paradigm demonstrated that IL-10 inhibits the activation of the p38 kinase by TLR4 in a STAT3-dependent manner through the activation of SOCS1 and SOCS3 [85]. Interestingly, STAT3, SOCS1 and SOCS3 are also activated by inflammatory stimuli such as LPS and TNF-α, and more importantly, SOCS activation can be induced by STAT3-independent mechanisms [94]. Therefore, it appears that TLR signals on the one hand promote cytokine production and on the other increase the pool of intracellular and intercellular negative regulators that inhibit further TLR signalling.

TLR-associated gene expression patterns in mucosal responses

Mucosal cells need to respond rapidly to microbial products to limit infection, restrain their functions before triggering tissue damage and chronic inflammation, and support the development of an adaptive immune response to microbial antigens. These features rely on the directness, rapidity, fine tuning and reversibility of gene expression patterns. TLR agonists are potent inducers of inducible transcriptional modules such as the inflammation-related family of the NF-κB transcription factors. The current knowledge on the requirement of the NF-κB family of proteins, its IκB inhibitors and its modulating kinases in TLR signalling and innate responses has been reviewed recently [95]. In this review, we wish to emphasize additional levels of TLR signalling that are of importance to innate responses.

Whereas transcriptional control defines the initial steps in the flow of genetic information, post-transcriptional mechanisms gradually and variably impose a series of flexible-rate limiting steps that modify protein synthesis in response to multiple environmental cues. Such post-transcriptional mechanisms encompass a number of steps affecting mRNA 'metabolism', including splicing, nucleocytoplasmic shuttling, stability and translational activation. It has been clear since the mid-1980s that myeloid cells stimulated by TLR4 agonists display discordant protein/mRNA profiles for induced cytokines [96, 97]. These observations were accompanied by the intriguing discovery that such cytokines contain small *cis* RNA sequence motifs in their untranslated termini that render them responsive to inflammatory signals [96]. One family of motifs that is highly conserved across mRNAs with relevance to mucosal immunity is the family of AU-rich elements (AREs). These elements are composed of a variable number of copies of the AUUUA or UUAUUUAUU nonamers and are currently classified into three structurally separable subgroups [98]. The first indication that ARE dysfunction can contribute to the development of mucosal inflammation came from studies on TNF-α ARE. The obligatory or conditional targeted deletion of TNF-α AREs in the mouse resulted in a severe IBD phenotype with resemblance to the human condition of Crohn's disease [90, 99]. In the absence of AREs, the LPS-induced TNF-α production from mouse macrophages was excessively prolonged, supporting a continuous state of innate activation as well as hypersensitivity to TLR agonists [85, 90, 99]. In molecular terms, these phenomena were attributed to the increased stability of the TNF-α mRNA and the absence of translational silencing mechanisms [85, 90].

Numerous ARE-binding proteins have been identified to date [98]. However, a few of these proteins have been implicated in the post-transcriptional control of inflammatory mRNAs in macrophages that thus could be involved in mucosal dysfunction. Tristetraprolin (TTP) is the prototype member of a family of zinc-finger-containing proteins; it is induced by LPS and binds to TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 AREs and promotes their degradation [100–102].

In TTP-deficient macrophages, the stability of TNF-α and GM-CSF mRNA is excessively prolonged, supporting a state of chronic inflammation [100, 101]. In contrast, a protein called HuR has emerged as an ARE-binding protein that acts to increase proinflammatory cytokine biosynthesis. HuR is the prototype member of the embryonic lethal abnormal vision (ELAV)-like family of RNA-binding proteins (RBP) [103]. Although in vivo evidence is currently lacking, in vitro experiments have indicated that HuR has pleiotropic functions on ARE-containing mRNA that include nuclear cytoplasmic shuttling, upregulation of mRNA stability and modulation of translational activation [104]. In terms of innate response, TLR4 engagement in macrophages increases the binding of HuR to TNF-α mRNA and increases its cytoplasmic stability [105]. More importantly, alterations of HuR binding to the ARE motif of TNF-α mRNA in lupus-prone mice correlate with reduced TNF-α production in response to bacterial stimulation and the development of autoimmunity [106]. Finally, two other ARE-binding proteins of interest are TIA-1 and TIA-R. In their absence TNF-α protein is overproduced, whereas steady-state mRNA levels remain unaltered, indicating that they are required to block the continuous translation of inflammatory mRNAs following LPS stimulation [107, 108].

How are these post-transcriptional modulators integrated into the signalling cascades emanating from TLRs? Studies on macrophages suggest that cytokine mRNA AREs respond to both positive and negative regulatory circuitries induced by TLRs. For example, LPS activates MAPK and SAPK pathways that in turn activate TNF-α, IL-8, IL-1 and COX-2 mRNA shuttling, stabilization and translation in an ARE-dependent manner [90–92, 109–111]. These signalling cascades can block the functions of negative ARE modulators like TTP and TIA-1 [112, 113]. These events may allow for the positive activities of HuR to enhance translational output, although direct evidence of such a mechanism is currently lacking. As soon as the LPS signal subsides, the TTP and TIA-1/TIA-R pools are activated to reduce proinflammatory production and restore homeostasis. Finally, it also appears that AREs are directly modulated by the cross-talk between TLRs and anti-inflammatory signals. To that end, a study on the modulation of LPS-induced TNF-α by IL-10 indicated that the JAK/STAT/SOCS pathway may directly inhibit the signals activating ARE-dependent translation [85], since in the absence of TNF ARE, TNF-α production was not inhibited by IL-10.

The evidence presented above provides only a rough road map of signals and gene expression modules implicated in mucosal responses, since most of our knowledge stems from analysis of individual TLR ligands on selective innate cell subpopulations. Currently, there is no clear indication as to whether these mechanisms are employed by the sum of TLRs and/or by activation of Nods in other cellular compartments of the mucosa. Future research in this area should provide important information on the organization of gene expression following selective or collective TLR engagement.

More to come

One long-standing question regarding the biological role of TLRs is whether and how they discriminate commensal versus potential pathogenic microorganisms, since all of them share the same microbial ligands, for instance LPS and unmethylated CpG motifs in bacteria. A recent study has shown that, at least in the gut, commensal bacteria are recognized by TLRs, and under normal conditions this interaction plays an important role in the maintenance of intestinal epithelial homeostasis [114]. Using oral administration of DSS to induce damage to colonic epithelium, the authors observed increased mortality and severe epithelial injury in MyD88-deficient mice, but not in wild-type mice. In addition, antibiotic depletion of microflora resulted in susceptibility of wild-type mice to DSS, a process that is reversed by oral administration of the TLR4 ligand LPS. These findings illustrate how the TLR/TLR ligand interactions are an important benefit to the host in intestinal homeostasis, on top of their critical role in sensing infection and triggering immunity.

Another unexpected finding that opens new avenues regarding our understanding of how TLRs direct mucosal immunity comes from the field of defensins. β-Defensins are small antimicrobial peptides of the innate immune system that are produced in response to microbial infection of mucosal tissue and skin [115]. It has been proposed that murine β-defensin 2 (mDF2β) acts as a so-called endogenous ligand of TLR4 on immature DCs. However, it is possible that mDF2β acts as a potentiator of subthreshold amounts of LPS that are tightly bound to it in a complex during defensin purification [116]. The question of how are these discriminating associations organize intracellular networks and gene expression modules is still far from answered, and it will most certainly require the cooperation of new disciplines from systems biology. Undoubtedly, further studies using innovative approaches are needed to evaluate in detail the various strategies that microbial molecules and PRRs use to elicit mucosal immunity and their critical role in mucosal homeostasis.

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