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REVIEW

Deciphering the complexity of Toll-like receptor signaling

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Abstract Toll-like receptors (TLRs) are essential players in the innate immune response to invading pathogens. Although extensive research efforts have provided a considerable wealth of information on how TLRs function, substantial gaps in our knowledge still prevent the definition of a complete picture of TLR signaling. However, several recent studies describe additional layers of complexity in the regulation of TLR ligand recognition, adaptor recruitment, posttranslational modifications of signaling proteins, and the newly described, autonomous role of the TLR4 co-receptor CD14. In this review, by using it as model system for the whole TLR family, we attempt to provide a complete description of the signal transduction pathways triggered by TLR4, with a particular emphasis on the molecular and cell biological aspects regulating its function. Finally, we discuss a recently reported model of CD14-dependent signaling and highlight its biological implications.

Keywords Toll-like receptor · TLR4 · CD14 · NFAT · Dendritic cell · Ubiquitin · Apoptosis · Innate immunity

Introduction

Although the seminal discovery of phagocytosis by Elie Metchnikoff dates back to 1883, the field of innate immunity has gained the appropriate attention only in recent years, thanks to Janeway's theory of innate immune recognition [1]. The cloning of the first Toll-like receptor (TLR), known as TLR4, together with the identification of its ligand, definitively established TLR4 as the long sought-after receptor for lipopolysaccharide (LPS) and paved the way for a whole new line of research in immunology, i.e. the identification of other members of the TLR family and the characterization of their signaling pathways. To date, more than a dozen of different TLRs have been identified, of which TLRs 1–9 are conserved among humans and mice, TLR10 is selectively expressed in humans, and TLR11 is functional in mice [2]. Although extensive research efforts have provided a considerable wealth of information on the biological functions and modes of action of TLRs 1–9 and 11, we still lack a basic knowledge of the physiology of the newly discovered family members TLRs 10, 12, and 13 (which, therefore, will not discussed further in this review). In the first part of this contribution, we review and update current knowledge on the structural biology and ligand recognition strategies of TLRs. In the following sections, we use TLR4 as model system for the whole TLR family and attempt to provide a comprehensive description of the signal transduction pathways triggered by TLR4, with a particular emphasis on the molecular and cell biological aspects regulating its function. Finally, we discuss a recently reported model of CD14-dependent signaling and highlight its biological implications.

Toll-like receptors

TLRs are type I transmembrane glycoproteins comprising an extracellular, transmembrane, and intracellular signaling domain, respectively. The extracellular domains of TLRs are responsible for ligand recognition and contain 16–28

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leucine-rich repeat (LRR) modules that provide the TLR ectodomains with a characteristic horseshoe-shaped folding [3]. Ligand binding by TLR ectodomains readily triggers homo-/hetero-dimerization between TLRs, resulting in the recruitment of different adaptor proteins to intracellular TIR [Toll/interleukin (IL)-1 receptor)] domains. The selective engagement of adaptors in turn defines the mode of signal transduction and, consequently, the biological outcome. Up to now, an impressive list of ligands for TLRs have been identified, including classical microbial products (bacterial, fungal, or viral), endogenous danger signals, and synthetic compounds. As we will provide here only a very brief overview of TLR ligand complexity, we refer the interested reader to other excellent reviews on the subject [2–4]. Together with TLR1 and TLR6, as well as the co-receptors CD14 and CD36, TLR2 recognizes a wide variety of microbial lipoproteins and plays a crucial role in the response to fungi and Gram-positive bacteria. Other TLRs with antibacterial activity are TLR5 and TLR9, which recognize flagellin and bacterial DNA, respectively, and TLR11, whose ligand specificity is poorly characterized. TLR3, TLR7, and TLR8 are important regulators of the antiviral response, a function that they exert though the recognition of single- and double-stranded RNA species. TLR4 is the most thoroughly studied TLR because of its outstanding role in antibacterial defense and its peculiar modes of signal transduction. It is well established that LPS from Gram-negative bacteria is the main ligand for TLR4, even though an effective immune response to LPS requires numerous additional players. Due to the massive body of information available on this sensing system, it will be specifically described and discussed in the following sections. The TLR family members can be classified into two groups according to their subcellular localization: TLR1, TLR2, TLR4–6, and TLR11 are expressed on the plasma membrane, whereas TLR3 and TLR7–9 are found in the endolysosomal compartment. Although this distinction is based on a mere phenotypical analysis, recent research has shown that it is highly informative of the functional role played by the two categories. Plasma membrane TLRs bind lipid or protein structures that are expressed on the surface of pathogens and are therefore readily available for recognition in the extracellular space. By contrast, the distinctive trait of intracellular TLRs is the shared ability to bind nucleic acids, which are normally confined inside an invading pathogen but can be encountered by immune cells upon phagocytic or endocytic events. It should be noted that the nucleic acid sensing strategy, although unavoidable since viruses lack conserved non-nucleic patterns, is not costless [5], as the inappropriate TLR activation by host DNA or RNA in pathologic setting may contribute to the emergence of autoimmune disorders. However, it has been shown that the intracellular localization of TLR9 is crucial for discriminating between self and non-self DNA, a hypothesis that may well be extended to the other nucleic acid-sensing TLRs [6]. Additionally, endolysosomal localization of TLR7 and TLR9 allows the latter to be proteolytically processed, a modification that renders them functional [7, 8]. A thorough cell biological approach has recently been used to study the features of TLR4 signaling, despite its apparently static localization on the plasma membrane. As it turned out, the functions of TLR4 also strictly rely on basic cellular mechanisms that regulate the ability of TLR4 to dimerize and recruit the adaptors MyD88 and TIR-domain-containing adapter-inducing interferon-β (TRIF) [5]. A detailed description of this process will be provided in the following sections of this review.

TLR4: a case study

Since its discovery, TLR4 has been the focus of much attention because of its peculiar features in terms of ligand recognition and signal transduction, two key aspects that this section will specifically deal with. TLR4 shows a highly orchestrated usage of co-receptors to discriminate between ligands, and this multifaceted receptor system additionally plays a role in triggering several signal transduction pathways through the sequential recruitment of at least four adaptor proteins. Since the resultant biological outcomes recapitulate the whole spectrum of TLR responses, we use TLR4 as a model to describe the molecular events linking receptor stimulation with downstream activation of transcription factors.

LPS structure

Lipopolysaccharide is a highly expressed component of the cell wall of all Gram-negative bacteria, and it plays a crucial role in maintaining the structural and functional integrity of the outer membrane [9]. Due to its vital biological importance, the general structure of LPS is conserved, making it a prototypical PAMP. LPS from most Gram-negative bacteria conforms to a general architecture composed of three separate regions, the lipid A, the core, and the *O*-chain. The lipid A moiety, which is highly hydrophobic, is largely responsible for the endotoxic activity of the whole LPS molecule. Lipid A is inserted into the external face of the outer membrane of the bacterial cell wall and is typically composed of a β-D-GlcN-(1,6)-α-D-GlcN disaccharide bearing two phosphates at positions $1'$ and $4'$, respectively. This structure is further modified by the attachment to the disaccharide module of up to four primary acyl chains, which can in turn be substituted by additional fatty acids. It appears that the lipid A structure associated with the highest

endotoxic activity is the *Escherichia coli*-like diphosphorylated β(1,6)-linked D-GlcN dimer with six acyl chains, as evidenced by the observation that deviations from this pattern invariably reduce the activity of the molecule [10]. Accordingly, the variant forms Eritoran, lipid IVa, and monophosphoryl lipid A act as antagonists or very weak agonists of TLR4:MD-2. The hydrophilic *O*-chain provides bacteria with an effective protection against hydrophilic antibiotics or complement proteins. It is a highly variable region composed of saccharidic units repeated up to 50 times, and a single organism will produce a wide range of these lengths due to incomplete synthesis of the chain. Since the *O*-chain is also generally targeted by antibodies, it is also referred to as *O*-antigen, and the serology of *O*-antigens has become an effective tool in typing strains of organisms and LPSs. Around 170 serotypes of *E. coli* LPS have been identified, demonstrating the high degree of variability of the *O*-antigen. However, the *O*-chain is not ubiquitous, as some Gram-negative strains seem to express a truncated version of LPS. Notably, bacteria carrying mutations in the genes involved in the synthesis and attachment of the *O*-chain do not express it at all. These mutants are called "rough" (R-) because of the morphology of the colonies they form in a plate, which is different from that observed for wild-type, "smooth" (S-)bacteria.

LPS-sensing machinery

Well before the definitive discovery of TLR4 as the transducing receptor for LPS, some of the key players involved in its recognition had already been discovered and characterized. After the isolation and cloning of LPS binding protein (LBP) [11], it was shown that its primary function was to extract monomers of LPS from aggregates or bacterial membranes [12] and facilitate LPS recognition by the receptor CD14 [13]. However, since CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein that lacks an intracellular domain for canonical signal transduction, it was evident that it could not function as the sole receptor for LPS [14]. The seminal discoveries of TLR4 [15, 16] and its associated co-receptor MD-2 [17] contributed to the establishment of a model in which CD14 acts as an LPS-sensing receptor that accepts LPS monomers from LBP and then transfers them to the TLR4:MD-2 complex, thereby promoting its ligand-induced dimerization.

LBP

LBP is a 58- to 60-kDa glycoprotein that is secreted in the serum mainly by hepatocytes as an acute phase protein. Its crystal structure can be inferred from that of the related permeability-increasing protein (BPI), which is composed of two barrel domains connected by a proline-rich linker, each accommodating a phosphatidylcholine molecule [18]. The LPS binding site of LBP consists of a cluster of cationic residues that bind the phosphorylated head of the lipid A moiety. The LPS binding site of LBP is fully exposed at the N-terminal tip of the protein, whereas the C-terminal domain is required for the interaction of LBP with the cell membrane or with CD14 [19]. At low concentrations, LBP has been shown to facilitate the recognition of LPS by extracting it as a monomer from aggregates or bacterial membranes [20]. LBP then catalyzes the transfer of LPS to both membrane-bound and soluble CD14 [13]. Notably, acute-phase high serum concentrations of LBP strongly inhibit LPS recognition, protecting the host from LPS- or Gram-negative bacteria-induced septic shock [21]. LBP is believed to exert this inhibitory role at least partly by transferring LPS to serum lipoproteins, such as high-density lipoprotein (HDL) [22] or by forming inactive aggregates with LPS [23].

CD14

CD14 is a 55-kDa glycoprotein expressed on the surface of myelomonocytic cells as a GPI-anchored receptor or secreted in a soluble form [24]. Sequence and crystallographic analyses revealed that CD14 contains 11 LRR modules folding into a truncated horseshoe-shaped tridimensional structure. Notably, CD14 has been crystallized as a dimer in which two monomers are bound by means of their C-terminal regions, and the total number of LRRs in the CD14 dimer is similar to that in TLR4, suggesting that the overall shape of the two proteins may coincide [25]. The concave surface is largely composed of β-sheets, whereas the convex face contains an irregular pattern of helices and loops. This structure implies the presence of a number of grooves and pockets that are crucial for ligand binding. The LPS binding site of CD14 is located at the N-terminal region of the protein in a large hydrophobic pocket, with a cluster of positively charged residues at the rim that accommodates the phosphorylated lipid A moiety [25]. Additionally, since also the carbohydrate portion of LPS contributes to the binding to CD14 [14], it has been proposed that the LPS binding site extends beyond the N-terminal pocket to include additional hydrophilic grooves in the neighboring area [25]. Such an irregular and flexible structure provides CD14 with the ability to bind different forms of LPS with a comparable high affinity and probably explains its ligand promiscuity. CD14 has indeed been involved in the recognition of a number of other ligands, acting as a co-receptor for TLR1, TLR2, TLR6, TLR4, and TLR3. CD14 functions as an LPS sensing receptor whose role is to enhance the sensitivity of innate immune cells to LPS by binding to picomolar

concentrations of LPS and facilitating its recognition by TLR4:MD-2 [26]. This was demonstrated by studies showing that CD14-deficient mice are highly resistant to LPS- or Gram-negative bacteria-induced septic shock [27] and that CD14-deficient macrophages display heavily impaired sensitivity to low concentrations of LPS [28]. Nevertheless, the detailed mechanism of how LPS is transferred to TLR4:MD-2 has not been completely unraveled. The simplest scenario would be that CD14 directly contacts TLR4:MD-2 and operates the LPS presentation. Alternatively, since CD14 has been consistently shown to catalyze the rapid insertion of LPS into the cell membrane [29], it is plausible to envisage a model in which CD14 leaves the LPS molecule in the cell membrane to be picked up by TLR4:MD-2. In support of both hypotheses is the identification of several residues of CD14 that are not required for LPS binding but for its signaling [30] and that could mediate the interaction with TLR4:MD-2. Furthermore, it has been shown that LPS is brought by CD14 in close proximity of TLR4:MD-2 [31] and that it is released before CD14 internalization [32]. Although its crucial role in the recognition of low LPS doses is established, CD14 has been shown to be largely dispensable for the response to high concentrations of LPS, which occur almost normally in CD14-deficient macrophages [28]. This notable observation suggests that an excess of LPS can be also sensed by means of a CD14-independent pathway, which possibly implies either direct LPS recognition by TLR4:MD-2 [33] or the participation of different LPS binding proteins [34]. In addition to its concentration, the chemotype of LPS determines the extent of CD14 requirement for ligand recognition. It has been demonstrated that the *O*-glycosylated S-form LPS typically triggers a response in terms of tumor necrosis factor alpha (TNFα) production that relies on CD14 activation, whereas CD14 is at least partially dispensable for the response to R-form LPS, which lacks a typical *O*-antigen [35]. This observation has been confirmed by a phenotypic screening of *N*-ethyl-*N*-nitrosurea-mutated mice, which showed that mice carrying a truncated version of CD14 (*Heedless*) lose the ability to activate the MyD88-dependent pathway and produce TNFα after stimulation with S-LPS but not with R-LPS or lipid A [36]. These data suggest that CD14 is essential for the presentation of S-LPS to TLR4:MD-2, whereas R-LPS or lipid A can be directly sensed by the receptor complex, which is consistent with other reports [33]. Strikingly, *Heedless* mice are also unable to produce type I interferons (IFNs), a TRIF-dependent class of cytokines, when challenged with both S- or R-LPS and lipid A [36]. Therefore, it appears that CD14 is strictly required for the induction of the TRIF-dependent signaling pathway irrespective of the LPS chemotype. Even though a different selection of each pathway by different LPS chemotypes has been observed [37], it should be stressed that the discrimination between LPS chemotypes seems to rely largely on TLR4:MD-2 rather than CD14 itself, since specific responses are observed even in the absence of CD14. The latter retains the ability to effectively bind both S- and R-LPS and its physiological expression nullifies their distinction in terms of signaling [36]. It has been proposed that CD14 specifically promotes TRIF-dependent signaling by acting as a chaperone or by inducing a conformational change in TLR4:MD-2 that results in the engagement of TRIF [36]. Regardless of the molecular mechanism, the observation that CD14 selectively regulates the production of type I IFNs can be interpreted as a major objection to the long-standing dogma that CD14, as a GPI-anchored receptor, can not have a direct signaling capability. As a matter of fact, this theory needs to be revisited, also in light of recent reports showing that various GPI-anchored proteins, exemplified by CD59, transiently recruit and activate downstream kinases after antibody-mediated cross-linking [38, 39]. Notably, GPI-lated CD14 itself has been demonstrated to trigger a phospholipase C (PLC)-dependent calcium mobilization following treatment with cross-linking antibodies [40]. Even though the biological significance of these findings could be argued due to the artificial clustering of the GPI-anchored proteins, we recently established a direct signaling role for CD14 in triggering a dendritic cell (DC)-specific pathway after recognition of its natural ligand S-LPS [41]. The features of this CD14-dependent and TLR4-independent signaling pathways will be described in detail in the last section of this review.

MD-2

MD-2 is a 25- to 30-kDa protein glycoprotein that interacts with TLR4 [17]. Among all of the TLR4 accessory molecules, MD-2 is the only one that is absolutely required for the response to LPS, as evidenced by the observation that MD-2- and TLR4-deficient mice display the same LPS unresponsive phenotype [42, 43]. Even though it has been demonstrated that MD-2 is the LPS binding component, it is not clear whether TLR4 may also directly bind LPS, as suggested by genetic evidence and recent structural studies [33, 44]. The crystal structure of MD-2 has recently been solved, both alone and complexed with TLR4 [44–46]. MD-2 is a β cup-folded protein with two antiparallel β-sheets that are separated on one side, with the internal hydrophobic core forming a large internal pocket, lined with cationic residues, which can accommodate LPS. The interaction between MD-2 and TLR4 relies on hydrogen and electrostatic bonds between two complementary charged patches located on each molecule [45] that have been identified as crucial for the formation of the

TLR4:MD-2 complex. MD-2 is instrumental for ligand-induced receptor dimerization, as confirmed by the recently reported structure of a crystallized TLR4:MD-2:LPS complex clearly revealing that, upon LPS binding, a symmetric "m"-shaped multimer composed of two TLR4:MD-2 heterodimers is formed [44]. The hydrophobic pocket of MD-2 accommodates five acyl chains of LPS, with the remaining chain exposed to interact with a secondary TLR4 by means of hydrophobic interactions. The phosphate groups also contribute to the dimerization by interacting with cationic residues of primary MD-2 and TLR4 as well as with secondary TLR4 [44]. Additionally, LPS binding induces a localized structural change in MD-2, triggering the protrusion of its Phe126 loop that makes hydrophilic contact with a secondary TLR4 [44]. In light of these recent findings, it seems that many local forces and interactions determine the extent of receptor dimerization and the resultant signaling. This experimental framework will be essential to systematically investigate how the TLR4:MD-2 system discriminates between different LPS molecules and how specific recognition is achieved.

Accessory molecules and structures

Despite the crucial importance of the LBP:CD14:TLR4:MD-2 system described above, an LPS-sensing machinery composed of such a small number of proteins may be an oversimplification that hardly explains the whole complexity of the LPS response. How a system will react to LPS may indeed vary significantly according to a number of parameters, including the host species, the cell type or the cell differentiation/activation state, and the nature, concentration, or duration of the stimulus. Additionally, the toxicity of an uncontrolled LPS response inevitably requires a panel of negative regulators. A growing list of accessory molecules, either positively or negatively involved in LPS recognition, has been identified that may compose a combinatorial cluster whereby the differential usage of co-receptors results in a specific LPS response [34]. Such a list includes the important negative regulators RP105:MD-1, TIR8, and ST2, whose function in LPS recognition has been described elsewhere [47]. Instead, we will focus on β_2-integrins and lipid rafts, as recent data suggest that these molecules/structures play a major role in LPS sensing as well as in the cell biological regulation of TLR4 signaling. The complement receptors CR3 (also called Mac-1 or CD11b/CD18) and CR4 (CD11c/CD18) are transmembrane glycoproteins that belong to the β_2-integrin family [48]. In addition to their role in cell adhesion, migration, and phagocytosis [48], CR3 and CR4 have been reported to bind LPS and to trigger LPS-induced NF-κB activation after heterologous expression in the TLR4-expressing CHO cell line [49, 50]. Direct interaction between CR3 and CD14 has also been shown in neutrophils stimulated with LPS in the presence of serum or LBP, implying a receptor crosstalk [51]. This observation led to a model of CR3 function in which LPS-induced CD14 clustering increases the local concentration of LPS and CR3, thereby potentiating the binding of LPS to CR3. This in turn results in a marked increase of CR3 adhesive properties, bridging bacteria to the surface of phagocytes [20], and in the subsequent detachment from CD14 [51], which then transfers LPS to TLR4:MD-2. Notably, CR3-mediated adhesion and phagocytosis that are up-regulated by LPS require its cytoplasmic domain, whereas a tail-less CR3 mutant can still contribute to NF-κB activation [50]. This suggests that CR3 may function, similarly but less efficiently than CD14, by presenting LPS to TLR4:MD-2. However, the significance of CR3 in mediating the cellular response to LPS has been questioned by a study reporting that CD18-deficient human monocytes and macrophages respond normally to LPS treatment in terms of TNFα and IL-1β production [13]. These data are in agreement with data reported elsewhere showing that monoclonal antibodies against CD11b or CD18 do not impair TNFα release by human monocytes [52, 53]. On the other hand, CR3 has been shown to be important for the production of TNFα by membrane-bound, particulate LPS [53] as well as for the expression of a panel of LPS-induced genes, including COX-2, IL-12p35, IL-12p40 [54], and IL-6 [55]. Another report also points to a crucial role for CD11b in regulating LPS signaling in macrophages, whose implications will be described below [55]. Lipid rafts are nanoscaled, spatially defined, and discrete assemblies of sphingolipid and cholesterol that fluctuate in lipid bilayers [56]. A key feature of lipid rafts is that they selectively incorporate proteins, such as GPI-anchored, doubly acylated, cholesterol-linked, and palmitoylated proteins; but a number of transmembrane proteins have also been reported to show a significant raft affinity [57]. Therefore, lipid rafts act as a signaling platform by increasing the local concentration of receptors and downstream effectors. Recent studies include the LPS-sensing molecules in the list of receptors whose activity is regulated by their localization into microdomains. As a GPI-anchored protein, CD14 normally resides in lipid rafts together with hsp70 and hsp90 [14, 58]. Upon LPS stimulation, TLR4:MD-2, CXCR4, and GDF5 as well as MAPKs are transiently recruited and segregated to microdomains, as determined by biochemical and FRAP (fluorescence recovery after bhotobleaching) measurement [59, 60]. Consistent with these observations, pharmacological disruption of raft integrity sensibly impairs the cellular response to LPS in terms of cytokine production [59]. Notably, we have recently shown that the sub-localization of CD14 in lipid microdomains is crucial for its own TLR4-independent signaling induced by LPS [41].

As described above, a conspicuous number of regulators of TLR4 signaling have been identified that exert their function by acting at each step of receptor activation. Ligand binding affinity, receptor sublocalization, clustering, and dimerization as well as downstream effector recruitment and activation are all subjected to both positive and negative forces that altogether shape the host response to LPS. In light of the complexity of this recognition system, further experiments are required to fully appreciate the relative contribution of each participant and to characterize how the interplay between them relates to the different, highly specific response triggered by bacterial infections.

TLR4 signaling pathways

Upon ligand binding, TLRs trigger several signaling pathways that culminate in the activation of specific transcription factors. Independently of the nature of the pathogenic stimulus, each TLR stimulates the production of proinflammatory cytokines (e.g. TNFα, IL-6, proIL-1β) whose transcriptional regulation depends on NF-κB and AP-1. All TLR family members, with the exception of TLR3, signal NF-κB and AP-1 activation by means of the adaptor MyD88. In the case of TLR3, the same biological output is obtained through the usage of a different adaptor, TRIF. Whereas the activation of NF-κB and AP-1 is a common feature of the signaling induced by all TLRs, only a subset of the latter is additionally able to trigger the production of type I INFs (IFN-α and IFN-β), a class of cytokines with potent antiviral and antibacterial activities. Indeed, only the intracellular, nucleic acid-sensing TLRs (TLR3, TLR7, TLR8, and TLR9) can activate the transcription factors IRF3 and IRF7, which largely regulate the expression of type I IFNs. This implies that the induction of type I IFNs is TRIF-dependent for TLR3, whereas it is MyD88-dependent for TLR7, TLR8, and TLR9. A notable exception to the rules outlined above—namely, (1) a single TLR can signal through either MyD88 or TRIF and (2) only intracellular TLRs trigger a type I IFN response—is represented by TLR4. TLR4 recruits both MyD88 and TRIF to induce the activation of NF-κB and AP-1 and, in a manner similar to TLR3, it uses TRIF to stimulate the production of type I IFNs, although in response to non-nucleic acid ligands. Furthermore, TLR4 requires the two additional upstream adaptors TIRAP and TRAM, which mediate the recruitment to TLR4 of MyD88 and TRIF, respectively. TLR1, TLR2, and TLR6 (but not TLR5, TLR7, TLR8, and TLR9) share the usage of TIRAP as a MyD88 bridging adaptor, whereas TRAM is specifically recruited to TLR4 (and not TLR3). These features of TLR4 effectively synthesize the complexity of TLR signaling, making it a prototype for the whole family. The next section will deal with the signaling pathways emanating from TLR4 in response to LPS, describing the modes of adaptor recruitment as well as the downstream cascades that lead to the biological outcome.

TLR4 adaptor recruitment

Originally shown to mediate IL-1R-dependent activation of NF-κB [61, 62], MyD88 is now known to play a crucial role in the signaling pathways induced by almost every TLR [63]. MyD88 has a modular structure, which consists of a C-terminal TIR domain that is responsible for the interaction with the TIR domain of recruiting TLRs, an intermediate domain, and an N-terminal death domain (DD) that performs homotypic interactions with downstream proteins of the IRAK family [61, 62]. Whereas MyD88 can be directly recruited to some TLRs (TLR5, TLR7, TLR8, and TLR9), it is not able to efficiently interact with TLR4 (and TLR1, TLR2, and TLR6), probably due to a lack of electrostatic complementarity between the respective TIR domains [64]. Therefore, TLR4 (and TLR1, TLR2, and TLR6) requires the intermediate adaptor TIRAP, also known as MAL, to bind MyD88 [65, 66]. Limited to TLR4 and TLR2 stimulation, the phenotypes of MyD88- and TIRAP-deficient mice are largely overlapping, with a completely abolished proinflammatory cytokine production that demonstrates a crucial role for TIRAP upstream of MyD88 in signaling by TLR4 and TLR2, but not by TLR9, IL-1R, or IL-18R [67, 68]. However, despite their total inability to produce proinflammatory cytokines, a reduced and delayed activation of NF-κB and AP-1 is still detectable in MyD88- or TIRAP-deficient mice after TLR4, but not TLR2, stimulation [63, 67, 68]. This late wave of NF-κB activation, as well as the expression of type I IFN genes, is a hallmark of the MyD88-independent signaling pathway that is specifically triggered by TLR4 by means of the adaptor TRIF, also known as TICAM-1 [68, 69]. Accordingly, TRIF-deficient mice do not produce measurable amounts of proinflammatory cytokines in response to TLR4 or TLR3 stimulation, but the early activation of NF-κB is still observed after LPS treatment. Any response to LPS is instead totally eliminated in mice doubly deficient for MyD88 and TRIF, showing that TRIF accounts for the MyD88-independent aspect of TLR4 signaling. Furthermore, no detectable expression of type I IFNs induced by TLR4 or TLR3 is observed in TRIF-deficient mice [70, 71]. Additionally, a fourth adaptor, TRAM (or TICAM-2), is specifically required for TLR4 to recruit TRIF and thereby promote TRIF-dependent NF-κB and IRF3/7 activation [72], two biological processes that are inhibited by the TIR-containing protein SARM [73]. SARM is thought to negatively regulate TRIF, but not MyD88, by directly binding to it and to prevent the recruitment of key downstream

effectors. Since TIRAP and TRAM are TIR-containing proteins that do not possess any obvious effector domain (i.e., DD), they are believed to function as bridging adaptors for MyD88 and TRIF, respectively. This hypothesis has recently been experimentally validated by several studies that also provided a detailed molecular description of the early events triggered by LPS recognition [55, 74]. Opposite to the common view of TIR-containing adaptors as cytosolic proteins that are recruited to membranes by means of TIR–TIR interactions with dimerized TLRs, TIRAP is normally found at actin-rich membrane ruffles as well as in intracellular vesicles, and its localization is TIR-independent [55]. Instead, both its localization and its function rely on an N-terminal phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$]-binding domain that is essential for targeting TIRAP to the membrane microdomains where TLR4 is located [55]. Since MyD88 is cytosolic, the biological function of TIRAP is to recruit MyD88 by means of a TIR–TIR interaction and to sort it to specific $PI(4,5)P_2$-rich membrane microdomains where signaling can initiate [55]. Consistent with the role of TIRAP as a sorting adaptor, MyD88-deficient macrophages, in which the localization of TIRAP is normal, do not produce cytokines after LPS stimulation. Instead, forced localization of MyD88 to $PI(4,5)P_2$-rich membrane microdomains restores the response to LPS in TIRAP-deficient cells, demonstrating that MyD88 is the signaling adaptor [55]. $PI(4,5)P_2$ is a phospholipid that plays many critical functions in the cytoplasmic leaflet of the plasma membrane, of which it is a minor constituent (about 1%). Apart from being the source of the second messengers inositol 1,4,5-trisphosphate [$I(1,4,5)P_3$], diacylglycerol (DAG), and $PI(3,4,5)P_3$, it regulates several processes at the cell membrane, including endocytosis, exocytosis, actin polymerization and cytoskeletal rearrangements and enzyme activation [75]. Many of these functions are operated by virtue of its ability to recruit proteins that bear a suitable $PI(4,5)P_2$-binding domain, such as the PH domain. It is believed that $PI(4,5)P_2$ is concentrated in the lipid raft, although this claim is somehow controversial, and it can not be taken as a general assumption. Nevertheless, several reports have consistently shown that $PI(4,5)P_2$ is concentrated in nascent phagosomes and membrane ruffles [76, 77]. The observations that TLR4 relocates to lipid rafts after activation [59] and that the $PI(4,5)P_2$-binding protein TIRAP colocalizes with TLR4 [55] strongly suggest that $PI(4,5)P_2$ may indeed be found in TLR4-containing microdomains. Further evidence for this is provided by our discovery of a CD14-dependent pathway induced by LPS that promotes the activation of PLCγ2, whose substrate is $PI(4,5)P_2$ [41]. One of the mechanisms that can explain the existence of pools of $PI(4,5)P_2$ at the plasma membrane is the localized synthesis of this lipid through the phosphorylation of PI(4)P by a phosphatidylinositol 4-phosphate 5-kinase [PI(4)P5K]. Interestingly, it has been reported that ARF6, a known positive regulator of PI(4)P5K [77] is essential for the localization of TIRAP and for the resultant TLR4 signaling [55]. ARF6 activation is in turn inhibited by AIP1, a newly described ARF6-GAP [78]. Since β_2 integrins can promote $PI(4,5)P_2$ synthesis, at least partly, by activating ARF6 [48], the observation that CD11b-deficient macrophages show a defective TIRAP localization and an impaired IL-6 production in response to LPS suggests a model in which LPS recognition by CD11b, probably mediated by CD14 (see above), stimulates an ARF6-dependent acute synthesis of $PI(4,5)P_2$. This allows TIRAP to locally concentrate and sort a pool of cytoplasmic MyD88 to the membrane microdomains where activated TLR4 is recruited [55]. It should be noted that this mode of integrin function, although convincingly demonstrated, is not consistent with previous reports showing that a tail-less version of CD11b is unable to promote phagocytosis but still activates NF-κB after LPS stimulation [50]. However, these discrepancies may result from an unphysiological behavior of CD11b-transfected cells as well as from a differential membrane organization between the cell types (CHO and primary macrophages) used. Based on the observation that CD11b is the only β_2 integrin expressed in macrophages but that DCs also express CD11c, one might speculate that in the latter cell type the two integrins may act comparably, since both have been shown to bind LPS. Specific experiments will be required to test this hypothesis. Additionally, it is plausible that a similar MyD88 recruitment strategy might be shared by TLR2, which also requires TIRAP to signal MyD88-dependent activation of NF-κB. Consistent with this hypothesis, TLR2 also relocates to the lipid raft after stimulation [60], it uses CD14 as a co-receptor, and it is assisted by β_2 integrins in the recognition of Gram-positive bacteria [79]. The functional distinction of TIRAP and MyD88 acting as a sorting and signaling adaptor has recently been shown to hold true also for the TRAM–TRIF axis, even though the modes by which the two adaptor pairs are recruited to TLR4 vary significantly [74]. The resultant model that describes the early phases of LPS-induced signal transduction is in sharp contradiction with the long-standing dogma that TLR4 simultaneously induces the TIRAP–MyD88 and the TRAM–TRIF pathways from the cell membrane. Indeed, unlike TIRAP, TRAM is found both at the plasma membrane [80] and in the $Rab5^+$ early endosomal compartment in resting cells [74]. The peculiar localization of TRAM is totally TIR-independent, but it is due to an N-terminal bipartite domain that contains a myristoylation motif [74, 80] (amino acids 1–7) and a polybasic region (8–20) [74]. A careful deletion analysis has revealed that both halves of the bipartite motif are required for targeting TRAM to the plasma membrane but

that only the myristoylation site is necessary for endosomal localization [74]. A myristoylation-deficient TRAM (either by deletion of the first seven amino acids or by substitution of a crucial glycine residue) is indeed uniformly distributed in the cytosol [74, 80], whereas a TRAM version lacking the polybasic region selectively resides in early endosomes [74]. Strikingly, unlike the cytosolic or a strict plasma membrane version, the endosome-targeted TRAM is able to fully restore the production of TRIF-dependent cytokines, such as IL-6 and RANTES, in TRAM-deficient macrophages treated with LPS [74]. This notable observation suggests that the LPS-induced TRAM–TRIF signaling occurs from endosomes, rather than the cell membrane. In resting cells, TLR4 is found predominantly at the plasma membrane, but it also recycles to $Rab5^+$ early endosomes by means of a dynamin-dependent mechanism that is different from the one used by TRAM, whose localization is insensitive to dynamin inhibition [74]. Within 15–30 min of LPS stimulation, TLR4, together with CD14 and LPS, is actively endocytosed through a dynamin-dependent mechanism [74, 81, 82]. Although it has been reported that the main function of LPS-induced TLR4 internalization is to promote its degradation [81], the inhibition of TLR4 endocytosis results in a selective impairment of TRAM–TRIF signaling, as measured by interferon regulatory factor 3 (IRF3) activation and IFNβ production [74]. This suggests that, after LPS stimulation, plasma membrane TLR4 moves into endosomes to interact with TRAM, which in turn recruits TRIF to trigger MyD88-independent NF-κB and IRF3 activation. Not only do these findings demonstrate that the TIRAP–MyD88 and TRAM–TRIF signal transduction pathways originater at spatially separated cellular locations, but they also demonstrate that these pathways originate sequentially in time. Therefore, a new model for the initiation of TLR4 signaling can be envisaged (Fig. 1). Initial LPS recognition by CD14 at the plasma membrane triggers the recruitment of TLR4 into $PI(4,5)P_2$-rich lipid rafts, as well as the acute synthesis of $PI(4,5)P_2$ through CD11b- and ARF6-mediated activation of PI(4)P5K. Here, TIR–TIR interactions drive the recruitment to TLR4 of the $PI(4,5)P_2$-binding protein TIRAP, which in turn sorts MyD88 to TLR4. This leads to the MyD88-dependent early wave of NF-κB and AP-1 activation. Soon after LPS stimulation, dynamin-dependent receptor endocytosis is initiated. At this early phase of membrane rearrangement, a synthesis of $PI(4,5)P_2$ is observed, probably because it provides docking sites for cytoskeletal components that regulate actin capping and nucleation [76, 83]. However, a subsequent drop in the local concentration of this phospholipid is required for the severing and the cytosolic release of a vesicle [76, 83] containing LPS as well as TLR4 and CD14. Since this compartment is then devoid of $PI(4,5)P_2$, TIRAP detaches from the endosomal membrane, leaving the TIR domain of TLR4 available for interaction with TRAM. TLR4-bound TRAM acts as a sorting adaptor for TRIF, which is recruited to TLR4 at the endosome to signal late NF-κB and AP-1 activation as well as IRF3 nuclear translocation. As the early endosome matures to $Rab7a^+$, a splicing variant of TRAM, TAG, inhibits TRAM–TRIF signaling by competing with TRIF for binding with TRAM [84]. Instead of a TRAM bipartite motif, TAG has an N-terminal GOLD domain that targets it to the endoplasmic reticulum as well as early endosomes in resting cells. Upon LPS stimulation TAG moves to late endosomes, where it functions as a signal switch-off by displacing TRIF from TRAM and by promoting the endolysosomal degradation of TLR4 [81, 84]. Triad3A is an E3 ubiquitin ligase that also promotes TLR degradation, even though the mechanisms regulating its participation to TLR trafficking is unknown [85]. How LPS-induced TLR4 endocytosis is initiated and which receptor proteins govern it, what is the role of $PI(4,5)P_2$ in this mechanism, and how $PI(4,5)P_2$ metabolism is regulated remain key questions that will need to be addressed by specific experimental approaches. Additionally, the functional reason for TRAM to localize at plasma membrane instead of residing exclusively in early endosomes remains unclear. A likely explanation comes from the analysis of the N-terminal bipartite domain of TRAM. This unstructured motif is also found on several proteins that are known to shuttle between the plasma membrane and the endosomes [74]. The most notable of these is MARCKS, a protein that shows a particular affinity for acidic phospholipids as $PI(4,5)P_2$ or $PI(3,4,5)P_3$ [86]. Since the myristoyl group alone is not sufficient for anchoring proteins to the plasma membrane, a second signal is required for effective protein localization. Basic residues in the polybasic region of the N-terminal unstructured domain of MARCKS have been reported to interact with the negatively charged heads of three molecules of $PI(4,5)P_2$, and MARCKS has been proposed to contribute to the lateral sequestration of $PI(4,5)P_2$ in the cell membrane. According to this model, there are two ways to displace MARCKS and release $PI(4,5)P_2$ at the cell membrane, both of which result in the disruption of the electrostatic interactions between the two molecules: (1) a Ca^{2+}-activated calmodulin binds the polybasic region of MARCKS, and (2) phosphorylation of three serines in the effector domain by a protein kinase (PKC) [86]. Similar to MARCKS, membrane-bound TRAM preferentially colocalizes with TLR4 in $PI(4,5)P_2$-rich microdomains. Interestingly, a serine residue in the basic domain of TRAM is phosphorylated by PKCε after LPS stimulation, and this event has been shown to play an important role in TRAM signaling [87]. Since the initial co-localization with TLR4 allows TRAM to be co-endocytosed with the receptor and since phosphorylation by PKCε may well be important for their internalization, it is likely that this is a system for

maximizing TLR4–TRAM interactions in endosomes. It is also tempting to speculate that TRAM initially acts as a "pipmodulin" that concentrates $PI(4,5)P_2$ at the cell membrane and that, upon PKCε phosphorylation, it releases $PI(4,5)P_2$, favoring its lateral dispersion, a condition for effective endocytosis. Finally, a recent study has reported the striking ability of TLR2 to signal type I IFN production in response to viral ligands—although only in $Ly6C^{hi}$ inflammatory monocytes [88]. Receptor internalization has been shown to be crucial for TLR2-induced type I IFN production [88], suggesting that TLR2 also induces distinct signal transduction pathways from both plasma membrane and intracellular locations. However, since TLR2 does not use TRAM or TRIF as adaptors, it is plausible that a $PI(4,5)P_2$-consuming endocytosis as the one proposed for TLR4 may not function for TLR2, as this would result in TIRAP-MyD88 detachment. Still, this observation reinforces the idea that TLR signaling must be studied from a cell biological point of view in order to integrate innate immune responses with the cellular infrastructure in which they operate [5].

MyD88-dependent signaling pathway

General features of NF-κB and MAPK activation NF-κB and AP-1 are crucial transcription factors that regulate a plethora of biological functions under both physiological and pathological conditions, including cell proliferation, cell development, and inflammation. Since a great deal of research effort has focused on these key regulators of cell physiology in recent years, we refer the readers to other review articles for an extensive and comprehensive description of this subject [89, 90]. Briefly, the NF-κB family of transcription factors is composed of five members, namely, p50 (NF-κB1) and its precursor p105, p52 (NF-κB2) and its precursor p100, RelA (p65), RelB, and c-Rel; all of these share an N-terminal Rel-homology domain (RHD) that mediates homo- and heterodimerization as well as sequence-specific DNA binding. NF-κB transcriptional activity is largely regulated by subcellular localization, as in resting cells NF-κB dimers are typically sequestered in the cytosol by the "classical" IκB proteins, namely IκBα, IκBβ, and IκBε. Receptor stimulation triggers a rapid K48-linked ubiquitination of IκB proteins and their subsequent degradation by the proteasome, resulting in the release of NF-κB proteins that translocate into the nucleus to promote gene expression. IκB degradative ubiquitination is dependent on a previous site-specific phosphorylation event that is operated by an activated IKK complex, composed of two kinase subunits (IKKα and IKKβ) and a regulatory component (IKKγ/NEMO). IKKβ accounts for the largest part of kinase activity in canonical NF-κB signaling, with IKKα playing a minor role that is still sufficient to compensate for IKKβ deficiency. NEMO is absolutely required for classical NF-κB activation, since it interacts with both IKKs and it induces the formation of a hexameric $(NEMO)_2$:(IKKα:IKKβ)$_2$ complex upon stimulus-induced dimerization. The biochemical details of how IKK catalytic activity is achieved are still unclear, but it probably results from IKK *trans*-autophosporylation in the activation loop as a consequence of NEMO-mediated oligomerization of IKK complexes. Alternatively, this initial phosphorylation step may be operated by an upstream IKK kinase, namely TAK1, whose activation is linked to receptor stimulation. Regardless of the molecular mechanism of IKK activation, post-translational modifications other than phosphorylation (K63-linked polyubiquitination, see below) are necessary for recruiting NEMO to the activated receptor and thus linking extracellular signals to intracellular responses. AP-1 is a dimeric transcription factor that is composed of members of the Jun, Fos, Maf, and ATF subfamilies of basic leucine zipper (bZIP) proteins. AP-1 activation is regulated by various mechanisms affecting transcriptional expression, protein turnover, and dimer formation of AP-1 subunits. In this process, a crucial role is additionally played by post-translational modifications, namely, phosphorylation by MAPKs in the nucleus. AP-1 activation by inflammatory stimuli is mostly mediated by the JNK, p38, and ERK groups of MAPKs, which are in turn phosphorylated by the MAPK-kinases (MAPKK) MKK4/7, MKK3/6, and MKK1/2, respectively. A MAPKK-kinase (MAPKKK), namely, TAK1, which is directly linked to the signaling pathway emanating from the stimulated receptor, mediates upstream activation of MAPKK. It has to be stressed that whereas stimulus-induced MAPKKK activation may occur upon recruitment to the receptor complex at the plasma membrane, MAPKKs and MAPKs are localized in the cytosol, which is the cellular compartment where they can be most efficiently activated and where they can encounter substrates. Therefore, the cytosolic translocation of the receptor signaling complex is a prerequisite for MAPK cascade activation. As we will describe below, this represents a key signaling event whose tight regulation ensures a full biological response to TLR stimulation.

IRAKs The first effectors downstream of MyD88 are the IRAK family proteins (Fig. 2). Originally identified as signaling partners of IL-1R [91], IRAKs were later shown to play crucial roles in TLR-mediated responses as well (with the exception of TLR3). However, much of our current knowledge on how IRAKs work is still based on early observations of IL-1R signaling pathways. Four different IRAKs have been identified in humans and mice, all of them sharing a basic domain organization [92]. All IRAKs have an N-terminal DD, which is typically responsible for

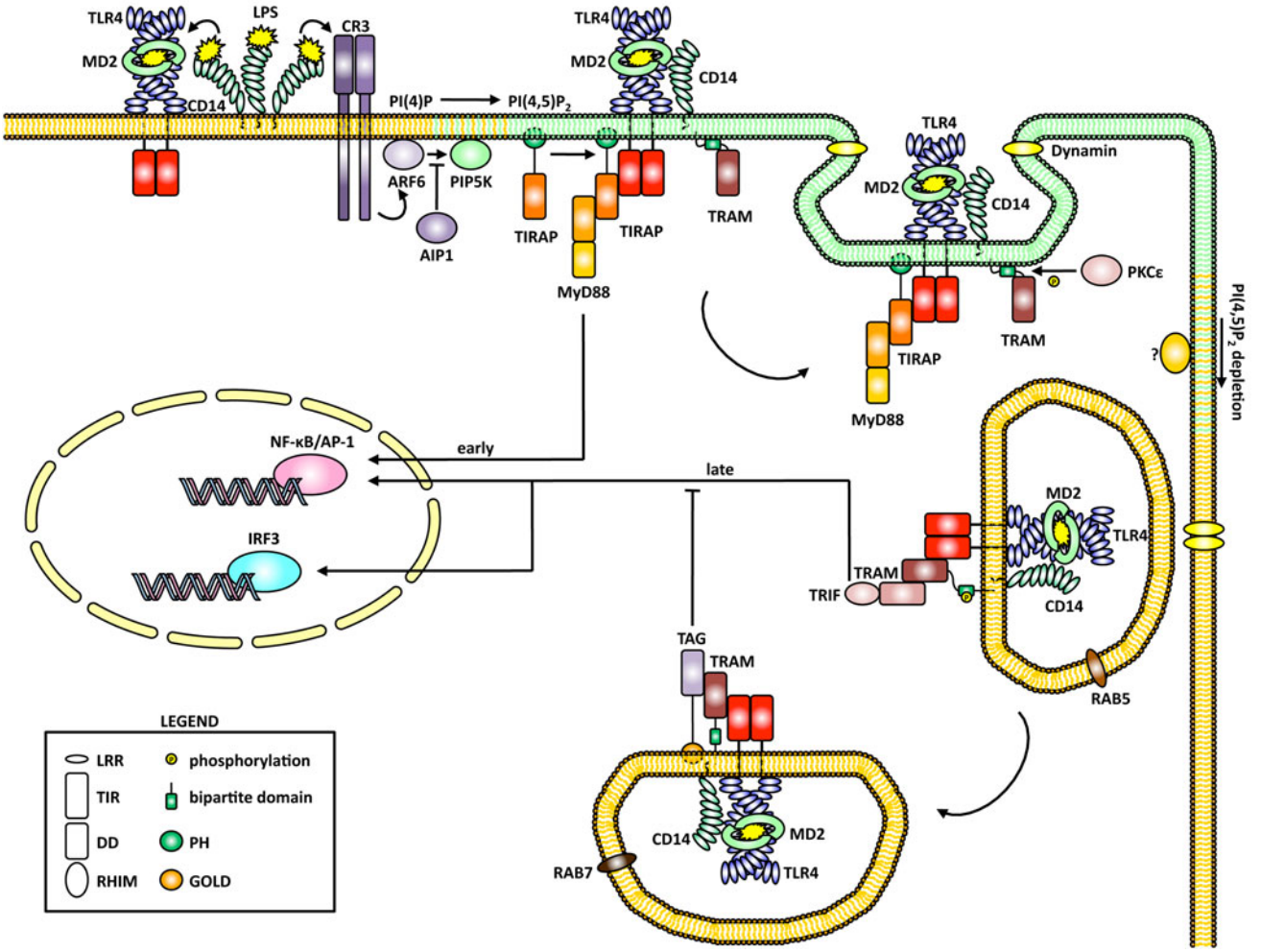

Fig. 1 Cell biology of the Toll-like receptor (TLR)4 adaptor recruitment. Upon lipopolysaccharide (LPS) recognition, the TLR4 co-receptor CD14 mediates endotoxin transfer to TLR4 and promotes its homodimerization. CD14 also triggers CR3 activation by LPS, resulting in a localized ARF6-dependent synthesis of $PI(4,5)P_2$ through phosphorylation of PI(4)P by PIP5K. Activation of the latter by ARF6 is negatively regulated by AIP1. Newly generated $PI(4,5)P_2$ allows TIRAP recruitment and consequent sorting of MyD88 to the activated TLR4:CD14 complex, which signals early NF-κB/AP-1 activation from the plasma membrane. LPS recognition by TLR4:CD14 also promotes receptor endocytosis through a dynamin-dependent process that is initially promoted by $PI(4,5)P_2$ synthesis but which requires $PI(4,5)P_2$ consumption for its termination. As a consequence of the drop in $PI(4,5)P_2$ concentration, whose regulation is currently unknown, TIRAP:MyD88 detach from $PI(4,5)P_2$-depleted $Rab5^+$ early endosomes, thereby freeing TLR4 for subsequent interaction with TRAM:TRIF. Due to its bipartite localization domain, TRAM resides both in early endosomes that fuse with TLR4:CD14-containing vesicles upon recycling and is coendocytosed with the receptor complex. This allows TRAM to sort TRIF to TLR4:CD14, resulting in the TRIF-dependent late wave of signaling to NF-κB/AP-1 and IRF3. As $Rab5^+$ early endosomes mature, TAG, which resides in $Rab7^+$ late endosomes, displaces TRIF from TRAM and thereby inhibits TRIF-dependent signaling

recruitment to MyD88, and a central kinase domain (KD) that differentially confers them the ability to function as serine/threonine kinases. Functionally, IRAKs transduce signals from the activated receptor to their downstream effector TRAF6, although each member plays a specific role in this process. IRAK-4 is the first protein to interact with MyD88, and it is the only IRAK that is absolutely essential for TLR/IL-1R signal transduction [93, 94]. Another feature that renders IRAK-4 unique among the family is that its biological activity strictly relies upon a functional kinase activity [93]. Indeed, IRAK-4-deficient mice show profoundly impaired responses to IL-1 as well as to TLR ligands [94], and overexpression of a kinase-deficient IRAK-4 in cells inhibits NF-κB activation [93]. IRAK-4 functions as a typical Ser/Thr kinase that, upon stimulation, phosphorylates the activation loop of IRAK-1 [93], thereby triggering a massive autophosphorylation of IRAK-1 [91, 95]. This phosphorylation event appears to be negatively targeted by the phosphatase SHP1, which therefore inhibits NF-κB and MAPK activation [96]. The recruitment of IRAK-1 to

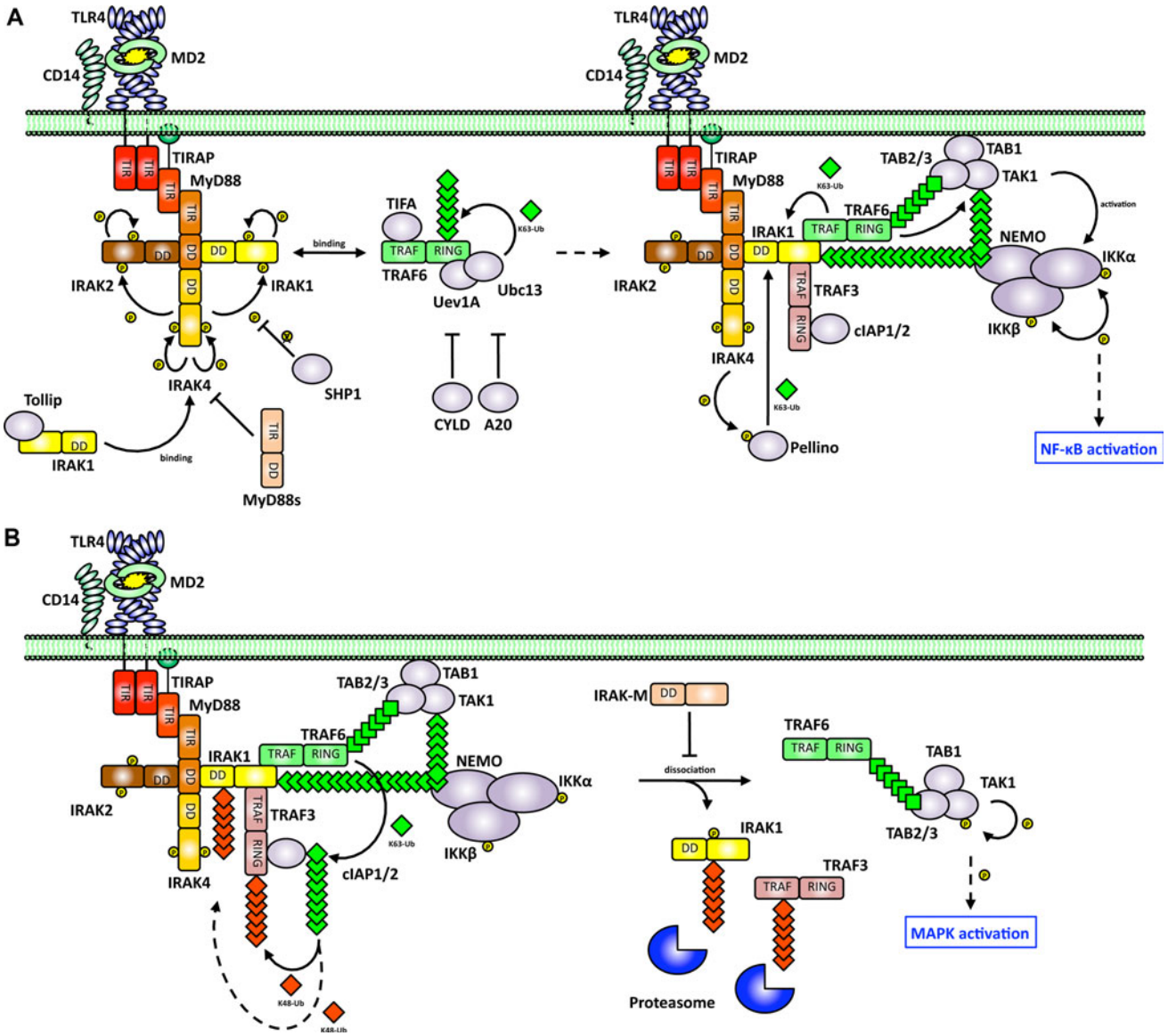

Fig. 2 MyD88-dependent NF-κB and AP-1 activation. **a** MyD88 mediates recruitment to TLR4 of IRAK4, IRAK1 (through Toll interacting protein, *Tollip*), and IRAK2 by means of a homotypic death domain (DD) interaction. This results in the activation of IRAK4 (by autophosphorylation), IRAK1, and IRAK2, which are initially phosphorylated by IRAK4 and then undergo additional autophosphorylation. Recruitment of IRAK4 and IRAK1 phosphorylation by IRAK4 are negatively regulated by MyD88s and SHP1, respectively. Phosphorylated IRAK1 shows an increased binding affinity for TRAF6, which is in turn recruited to the receptor. TIFA-dependent oligomerization of TRAF6 stimulates its E3 ubiquitin ligase activity and, in coordination with the E2 complex Uev1A:Ubc13, TRAF6 catalyzes the attachment of K63-linked polyubiquitin chains on a number of substrates, including itself, in a process that is inhibited by CYLD and A20. Ubiquitinated TRAF6 interacts with TAK1 via TAB 2/3. TRAF6 also promotes K63-linked polyubiquitination of IRAK1 and TAK1, which directly recruit NEMO to the receptor complex. Ubiquitination of IRAK1 may also be operated by Pellino, which is in turn activated by IRAK4 and IRAK1 itself (not shown). Upon co-recruitment at the receptor complex, TAK1 promotes IKKα/β activation through a process that is independent of TAK1 kinase activity and which occurs at the plasma membrane. This ultimately results in MyD88-dependent activation of NF-κB. Even if not shown, it has to be noted that IRAK2 is likely to behave similarly to IRAK1 in promoting sustained NF-κB activation after IRAK1 degradation (see below). **b** In addition to TRAF6, IRAK1 probably mediates the recruitment of TRAF3 and cIAP1/2 to the receptor complex where TRAF6 catalyzes K63-linked polyubiquitination of cIAP1/2. K63-linked polyubiquitinated cIAP1/2 is enzymatically active as an E3 ligase that promotes degradative K48-linked polyubiquitination of TRAF3 and possibly IRAK1. Upon subsequent proteasomal degradation of TRAF3 and IRAK1, the TRAF6-nucleated complex containing TAK1 dissociates from the receptor and is released into the cytosol in a process that is inhibited by IRAK-M. Once in the cytoplasm (the cellular compartment where TAK1 substrates are located), TAK1 triggers effective MAPK activation by initiating a cascade of phosphorylating events. The kinase activity of TAK1 is therefore absolutely required for cytosolic MAPK activation

MyD88 occurs with a slower kinetics than that of IRAK-4 due to the initial interaction of unphosphorylated IRAK-1 with the adaptor Tollip [97]. In resting cells, Tollip binds IRAK-1 in the cytosol and suppresses its kinase activity, consistent with an inhibitory effect of Tollip overexpression [97]. Upon activation, Tollip interacts with both TLR and IL-1R complexes, bridging IRAK-1 to MyD88 and favoring IRAK-1 signal transduction [98]. IRAK-1 and IRAK-4 do not form heterodimers [93], but they are brought into close proximity by means of MyD88, which acts as a signaling platform. Accordingly, in the presence of MyD88s, which is a shorter splice variant of MyD88 induced by LPS, IRAK-4 is not recruited to MyD88 and no IRAK-1 phosphorylation is observed [99]. Upon hyperphosphorylation, IRAK-1 weakens its binding affinity for MyD88, while increasing its ability to bind TRAF6 [61, 100]. This results in a rapid and transient recruitment of TRAF6 to a conserved C-terminal motif of IRAK-1, followed by their dissociation from the receptor [101]. IRAK-1 and TRAF6 initially remain at the plasma membrane, where TRAF6 interacts with a preassembled complex composed of TAK1, an important downstream effector kinase (see below), and the TAK1 binding proteins (TAB)1, TAB2, and TAB3 [101]. Catalytically active TAK1 is only observed in the cytosol, where TAK1, together with TAB1, TAB2, TAB3, and TRAF6, is released shortly after interacting with IRAK-1 [101]. The process regulating the cytosolic translocation of the putative TRAF6:TAK1:TAB1:TAB2:TAB3 multimeric complex is poorly defined, although it has been shown to depend on IRAK-1 [101, 102]. Interestingly, IRAK-1 itself does not move into the cytosol but remains at the cell membrane where it is degraded [95] through mechanisms that conflicting reports have reported to be either proteasome-dependent [95] or -independent [103]. In this regard it should be noted that IRAK-1 contains two PEST sequences in its UD region that may be involved in IRAK-1 degradation. It is plausible that IRAK-1 degradation functions as a system to promote the cytosolic release of the signaling complex containing TRAF6 and TAK1 and also as a negative feedback control that regulates excessive activation by TLR/IL-1R. Despite the model depicted above, possibly implying an essential role of IRAK-1 in inflammatory signal transduction, it is known that IRAK-1 alone is not sufficient in this process. IRAK-1-deficient mice are more resistant than wild-type mice to the toxic effects of LPS but their phenotype is much less evident than IRAK-4- or MyD88-deficient mice, also in terms of cytokine production [104]. This suggests that other IRAK family members may take over IRAK-1 functions and compensate for its absence. Additionally, although IRAK-1 is undoubtedly functional as a kinase, it has been reported that IL-1-induced activation of NF-κB in cells lacking IRAK-1 can be fully restored by transfection of a kinase-deficient version of IRAK-1 [105]. Therefore, rather than by phosphorylating substrates, IRAK-1 (and also IRAK-2 and IRAK-M, see below) appears to exert its role through protein–protein interactions, basically behaving as a signaling adaptor. Since its discovery, IRAK-2 has always been found to be able to share functional, other than structural, properties with IRAK-1. Similarly to IRAK-1, IRAK-2 interacts with both MyD88 and TRAF6, and its overexpression in cells activates NF-κB [62]. The two proteins seem to act redundantly, as IRAK-2 can largely restore IL-1-induced NF-κB activation in IRAK-1-deficient cells [105], and they can also form heterodimers. However, the physiological role of IRAK-2 has only recently been unveiled, thanks to the generation of gene-targeted mice [106]. The mechanical features of IRAK-2 function are comparable to those of IRAK-1, as also IRAK-2 is recruited to MyD88, where the initial phosphorylation by IRAK-4 triggers IRAK-2's own kinase activity and subsequent interaction with TRAF6 [106]. IRAK-2 differs from IRAK-1 in that its action is delayed, and it is important for sustaining NF-κB activation, rather than inducing it. Accordingly, cells from IRAK-2-deficient mice show an impaired cytokine production upon TLR/IL-1R stimulation as a result of a defective late-phase transcriptional expression [106]. This sustained activity of IRAK-2 is consistent with a prolonged half-life of the protein, which is not degraded upon activation. Interestingly, the UD region of IRAK-2 lacks the PEST sequences that are observable in IRAK-1 and that may regulate its destruction. Although IRAK-1 and IRAK-2 act in a kinetically distinct fashion, they can largely compensate for each other's functions. This is clearly demonstrated by the observation that genetic ablation of either IRAK-1 or IRAK-2 in mice generates only a partially impaired phenotype compared to mice doubly deficient in IRAK-1 and IRAK-2, which are almost totally unresponsive to TLR/IL-1R stimulation [106]. An additional feature of IRAK-2 is that its biological function seems to depend on an intact kinase activity [106], a notable observation because the kinase domain of IRAK-2 is predicted to be non-functional. The fourth member of the IRAK family is IRAK-M, a kinase-inactive protein that is selectively expressed in monocyte and macrophage populations [107]. IRAK-M has been reported to negatively regulate signaling through TLR/IL-1R by inhibiting the dissociation of IRAK-4 and IRAK-1 from the receptor [108]. IRAK-M expression is strongly increased upon LPS stimulation, and it seems to play a crucial role in endotoxin tolerance, as shown by the observation that pre-stimulated IRAK-M-deficient macrophages are not hyporesponsive to a second LPS administration [108].

TRAF6 The TRAF family comprises seven family members that play important roles in the signal transduction to NF-κB triggered by a number of receptors,

including TNF-R, IL-1R, TLRs, BCR, and TCR [109]. The distinctive feature of all TRAF proteins is a C-terminal domain, termed TRAF, which is in turn composed by an N-terminal coiled-coil region (TRAF-N) and a C-terminal β-sandwich (TRAF-C). The TRAF domain mediates protein–protein interactions, with TRAF-N regulating self-oligomerization and TRAF-C conferring binding to upstream molecules. Apart from TRAF1, all TRAFs contain a RING finger domain at their N-termini, followed by a variable number of zinc fingers. The effector function of the RING domain has been revealed by biochemical studies of TRAF6, which is the crucial TRAF protein for MyD88-dependent NF-κB activation [110]. TRAF6 acts as an E3 ubiquitin ligase that, in coordination with the E2 ubiquitin-conjugating complex Uev1A:Ubc13, promotes the attachment of lysine-63(K63)-linked polyubiquitin chains to several substrate molecules, including TRAF6 itself [111]. Notably, K63-linked polyubiquitination differs from the classical K48-linked polyubiquitination in that it does not constitute a signal for proteasomal degradation; rather, it functions as a signaling moiety in many NF-κB regulatory pathways. This regulatory role relies upon the ability of a number of signaling molecules to recognize and interact with K63-linked polyubiquitin chains through specific ubiquitin-binding domains [112]. Therefore, K63-polyubiquitinated proteins act as scaffolds that recruit downstream effector molecules by providing them with suitable docking sites. Consistent with a crucial role of this post-translational modification in NF-κB signaling, many proteins involved in this pathway have been shown to function either as acceptor or interacting partners for K63-linked polyubiquitin chains. Key targets for K63-linked polyubiquitination in the TLR/IL-1R-induced MyD88-dependent pathway include TRAF6 itself [111], IRAK-1 [103], and TAK1 [113], whereas TAB2 and TAB3 [114], and NEMO [115, 116] have been shown to bind K63-linked polyubiquitins through specific ubiquitin-binding domains. These data have led to a model of MyD88-dependent signal transduction whereby, upon TLR/IL-1R stimulation, TRAF6 is recruited by IRAK-1 at the cell membrane. Here, due to the clustering of receptors and adaptors, several TRAF6 molecules oligomerize via their TRAF domains in a process that is assisted by the TRAF6-interacting protein TIFA [117, 118]. Multimerized TRAF6 triggers its own E3 ubiquitin ligase activity and promotes its auto-K63-linked polyubiquitination. K63-linked polyubiquitinated TRAF6 directly binds to TAB2 and TAB3 [114] which, together with TAB1, interact with TAK1 at the cell membrane. Upon IRAK-1 degradation, this TRAF6-nucleated complex is released into the cytosol, where the interaction between TRAF6 and TAB2/TAB3 is thought to result in TAK1 oligomerization mediated by TAB2/TAB3, followed by TAK1 transactivation [114] (Fig. 2a). Oligomerization of proteins is a driving force for many signaling pathways because it allows the formation of platforms where auto- or cross-interactions can take place. The TLR/IL-1R pathway is no exception to this rule, as MyD88 and IRAK-4 have been shown to form large complexes with 7:4 or 8:4 stoichiometry, i.e., the "Myddosome" [119], and artificial clustering of MyD88 is sufficient to mediate NF-κB and AP-1 activation without the need for receptor stimulation [120]. Therefore, it should be kept in mind that this signaling pathway generally does not act linearly (protein A activates protein B) but relies rather on multiple, cooperative interactions that altogether bring molecules in close proximity to each other. In this context, K63-linked polyubiquitin chains represent a means for modified proteins to perform additional, long-range interactions. Despite the model of TLR/IL-1R-induced TAK1 activation being strongly based upon the role of K63-linked polyubiquitination of target proteins, direct experimental evidence supporting this assumption has been provided only recently. Various studies have reported conflicting data about the importance of Ubc13 for the activation of NF-κB, at least in some signaling pathways, suggesting the existence of additional E2 enzymes [121, 122]. The formal demonstration for an essential function of K63-linked polyubiquitination in NF-κB activation has also been hampered by technical difficulties in mutating the multiple ubiquitin genes in vivo. However, the recent generation of a tetracycline-inducible ubiquitin replacement system in human cells has ultimately shown that Ubc13-mediated K63-linked polyubiquitination is essential for NF-κB activation by IL-1β (and presumably also by TLRs) [123]. Also, the in vivo importance of the E3 ubiquitin ligase activity of TRAF6 has remained controversial. However, another recent report has shown that, in a cell-free system, the essential role of TRAF6 for TAK1 activation depends on its ability to generate free, unanchored K63-linked polyubiquitin chains that act as second messengers to promote TAB2/TAB3-mediated TAK1 oligomerization without the need for a direct interaction with TRAF6 [124]. This seminal study identifies a novel regulatory apparatus for activation of protein kinases by ubiquitin, and it suggests a mechanism for NF-κB activation in vivo. Regardless of the molecular details of how K63-linked polyubiquitin polymers participate in cell signaling, the biological relevance of this post-translational modification is underlined by the existence of K63-specific deubiquitinating enzymes. A20 is a crucial regulator of the inflammatory response, a role that it exerts by deconjugating K63-linked polyubiquitin chains from TRAF6 and RIP1 [125]. Downstream of TLRs, A20 inhibits the E3 ligase activity of TRAF6 by promoting degradative K48-linked polyubiquitination of the E2 enzymes Ubc13 and

UbcH5c [126]. It is possible that a similar biological function is played by CYLD, which is likely to act redundantly with A20 [127]. TRAF3 is another MyD88-interacting TRAF protein [120, 128] whose function in TRIF-dependent TLR4 signaling is well established (see below). However, how it works in MyD88-dependent TLR4 signaling has remained elusive until recently. Differently from TRAF6, which promotes both NF-κB and MAPK activation, TRAF3 has been shown to play no role in NF-κB signaling but to act as a negative regulator of MAPK activation through CD40 [129] and TLR4 [130]. MAPK signaling occurs in the cytosol and depends on prior intracellular translocation and consequent activation of TAK1, which acts as a MAPKKK in TLR signaling (Fig. 2b). Upon recruitment to the activated receptor complex at the plasma membrane, TRAF3 exerts its negative function by preventing the cytosolic release of the TRAF6-nucleated signaling complex containing TAK1 [130]. Either directly or indirectly, MyD88 also recruits the E3 ubiquitin ligases cIAP1 and cIAP2, which specifically catalyze the transfer of K48-linked polyubiquitin chains to TRAF3 [129, 130]. K48-linked polyubiquitination of TRAF3 by cIAP1/2 serves as a signal for its proteasomal degradation, an essential event for the detachment of the TAK1-containing signaling complex from the cell membrane and the subsequent TAK1 activation. How TRAF3 is recruited to MyD88 is unclear, but it is plausible that, similarly to TRAF6, it interacts with IRAK1 at the cell membrane. Since IRAK-1 has been proposed to undergo both non-degradative K63- (see below) and degradative K48-linked polyubiquitination through a process of ubiquitin editing [131], this hypothesis raises the possibility that cIAP1/2 may also promote IRAK-1 degradation through K48-linked polyubiquitination (Fig. 2b). This would provide a mechanistic explanation for the cIAP1/2-dependent dissociation of the TRAF6-nucleated, TAK1-containing signaling complex. It is notable that cIAP1/2 catalytic activation depends on their previous K63-linked polyubiquitination mediated by TRAF6 [130], which therefore links TLR4 stimulation with the relieving of TRAF3-mediated inhibition of MAPK signaling. Although neither E3 ubiquitin ligase activity nor K63-linked polyubiquitination of TRAF3 are observed in MyD88 signaling, TRAF3 works differently in TRIF-dependent signaling, where its alternative, non-K48-linked polyubiquitination provides it with an important signaling function.

TAK1 TAK1 is a crucial signaling intermediate in TLR/IL-1R and TNFα signaling to NF-κB and AP-1 [132], a function that derives from its ability to induce IKK as well as MAPK activation [133]. Therefore, TAK1 constitutes the point at which the MyD88-dependent pathway diverges into the NF-κB and AP-1 signaling branches. These biological processes regulated by TAK1 strictly rely on co-expression of TAB 1, TAB 2, and TAB 3, since overexpression of TAK1 alone does not result in significant NF-κB activation [133, 134]. TAB 1 aids in TAK1 autophosphorylation, acting as an activating subunit in the TAK1 complex [135], whereas TAB 2 and TAB 3 play redundant roles in facilitating TAK1 recruitment to TRAF6 through specific binding to K63-linked polyubiquitin chains. The physiological importance of TAB 2 and TAB 3 in TLR signaling is underlined by the observation that the function of these two proteins is negatively regulated by the RING finger-containing TRIM30α [136]. TRIM30α promotes TAB 2 and TAB 3 degradation through the endolysosomal compartment, rather than by catalyzing K48-linked polyubiquitination [136]. Although TAK1 is undoubtedly required for NF-κB activation by proinflammatory stimuli, the mechanism by which TAK1 activates IKK is still unclear. TAK1 has been shown to function in vitro as an IKK-K by phosphorylating IKKβ in its activation loop [133]. In light of this observation, a model of TAK1-mediated IKK activation has been proposed in which TRAF6 K63-linked polyubiquitin chains recruit both TAK1 and NEMO, as NEMO contains an ubiquitin-binding domain named NUB that confers specificity for K63-linked polyubiquitin chains and is required for NF-κB activation [115, 137] (Fig. 2a). Through this interaction, NEMO brings the catalytic subunits IKKα and IKKβ into close proximity with TAK1 at the receptor complex, thereby facilitating IKK activation through direct phosphorylation by TAK1. Notably, TAK1 itself has been recently reported to undergo TRAF6-mediated K63-linked polyubiquitination, a modification that is important for IKK recruitment [113]. As anticipated above, IRAK-1 is also a substrate for K63-linked polyubiquitination, and modified IRAK-1 interacts with NEMO to promote NF-κB activation [103, 138]. The E3 ubiquitin ligase responsible for K63-linked polyubiquitination of IRAK-1 has been identified as TRAF6 [138] or Pellino isoforms [139] in conflicting studies. Importantly, Pellinos appear to be activated through phosphorylation by IRAK-1 and IRAK-4, whose kinase activity might at least be initially required to promote IRAK-1 K63-linked polyubiquitination. All of these observations suggest that TAK1-dependent IKK activation occurs at the plasma membrane through the concomitant recruitment of TAK1 and IKK at the receptor complex. However, formal demonstration for the existence and the biological significance of TAK1 phosphorylation of IKKβ in vivo is still lacking. Instead, several pieces of evidence point to a role of TAK1 in the activation of IKK that is independent of its kinase activity. Catalytically active TAK1 is only observed in the cytosol [101] after cIAP1/2-dependent detachment of TAK1-containing complex from the plasma membrane [129, 130], whereas IKK activation is thought to occur at the cell surface.

Accordingly, inhibition of the cytosolic translocation of this complex by interfering with cIAP1/2 functionality completely blocks TAK1 catalytic activation, as measured by MAPK phosphorylation, but has no consequence on IKK activation, which proceeds normally [130]. These results strongly suggest that, whereas MAPK signaling is strictly dependent on TAK1 kinase activity, activation of NF-κB relies on TAK1 functions other than phosphorylation (Fig. 2b). As the relative importance of K63-linked polyubiquitination of the specific proteins involved in IKK activation is difficult to ascertain, due to a certain degree of redundancy between potential scaffolding adaptors, all of these observation are consistent with a model whereby K63-linked polyubiquitin chains act as second messenger to generate a complex molecular net that facilitates protein–protein interactions. Further complicating the picture is the discovery that additional E2 enzymes with different ubiquitin linkage specificity may participate in NF-κB activation. The E2 UbcH5c can interact with TRAF6 to synthesize ubiquitin polymers containing both K48 and K63 linkages that trigger IKK activation by NEMO recruitment [124]. Interestingly, linear head-to-tail polyubiquitin chains can activate NF-κB by binding to another ubiquitin-binding region of NEMO [140] or by modifying NEMO itself [141], even though this type of ubiquitin polymer has been reported to inefficiently activate IKK in vitro [124]. Differently from its role as an IKK-K, TAK1 function as a MAPKKK is solidly documented. Upon cytosolic activation, TAK1 phosphorylates crucial substrates that in turn promote the activation of MAPK signaling. TAK1 has been reported to phosphorylate MKK3/6 [133] and MKK4 [142], thereby promoting the subsequent activation of p38 and JNK, respectively. ERK activation, which is regulated by MKK1/2, is believed to occur independently of TAK1 but to rely on Tpl2. Additional MAPKKK whose activation is induced by TLRs and which may contribute to classic MAPK signaling include MLK2 and MLK3, at least for p38 and JNK [143]. Finally, MEKK3 is an important MAPKKK that is believed to function similarly to TAK1 in TNFR-induced NF-κB and MAPK signaling [144]. An important role for MEKK3 has also emerged in TLR4 and IL-1R signaling, as its deficiency abrogates NF-κB as well as p38 and JNK MAPK activation [145]. MEKK3 is recruited to TRAF6 upon receptor stimulation, and it appears to function at the same hierarchical level of TAK1, albeit with a delayed activation kinetics. This partial redundancy may explain why the requirements for TAK1 in promoting NF-κB and MAPK activation vary significantly from cell type to cell type [132]. However, the molecular mechanisms regulating MEKK3 activation remain to be determined, particularly with respect to the role of K63-linked polyubiquitination of MEKK3.

TRIF-dependent signaling pathways

RIP1-dependent NF-κB activation The adaptor TRIF is structurally different from MyD88 in that it does not contain a DD but has an N-terminal domain that has been reported to directly bind TRAF6 to promote NF-κB activation [146]. However, several studies in TRAF6-deficient cells have contradicted this observation, raising the question of the importance of TRAF6 in this biological process [110, 120]. Instead, the key player for TRIF-dependent NF-κB activation induced by TLR3 and TLR4 is the kinase RIP1, which interacts with TRIF via a RHIM domain that is located at the C-terminal region of TRIF [147] (Fig. 3). This process is inhibited by RIP3, a related, inactive protein that competes with RIP1 for TRIF binding [147]. Proinflammatory cytokine, but not type I IFN production is impaired in RIP1-deficient cells after TLR3 or TLR4 stimulation, showing a selective involvement of RIP1 in NF-κB activation [147, 148]. RIP1 is known to play a crucial role in TNFR-induced NF-κB activation, where it is recruited to the receptor complex through a homotypic DD interaction with the adaptor TRADD [149, 150]. Via its intermediate domain, RIP1 then binds the E3 ubiquitin ligase TRAF2/5 that, analogously to TRAF6, catalyzes the K63-linked polyubiquitination of RIP1 and the subsequent recruitment of NEMO [115]. Polyubiquitinated RIP1 also interacts with TAK1 via TAB2 [114], and with MEKK3, which in turn activates IKKs. Recent studies have reported that the way in which RIP1 drives TRIF-dependent signaling to NF-κB after TLR stimulation is reminiscent of what has been described for TNFR. After TLR stimulation, TRIF directly recruits RIP1, which in turn interacts with the adaptor TRADD via a homotypic DD interaction [149, 150]. RIP1 then undergoes K63-linked polyubiquitination [148] independently of TRAF6, a process that facilitates IKK activation through TAB2:TAK1 recruitment. TRADD is functionally involved in TRIF-dependent NF-κB activation, as TRADD-deficient cells are impaired in their ability to produce proinflammatory cytokines upon LPS or poly(I:C) stimulation [149, 150]. How TRADD acts at a molecular level in TLR signaling is still unclear, as TRAF2, which is a typical TRADD binding partner, plays no role in this pathway. It is tempting to speculate that TRADD may act as a scaffolding adaptor for an E3 ubiquitin ligase different from TRAF6 that mediates K63-linked polyubiquitination of RIP1. In this regard, a recent report has identified Pellino1 as a non-redundant E3 enzyme that, upon interaction with RIP1, catalyzes its post-translational modification and regulates NF-κB activation [151] (Fig. 3).

In addition to the pathway described above RIP1 can also promote NF-κB activation through a different mechanism involving FADD and caspase 8 (Fig. 3). The FADD-

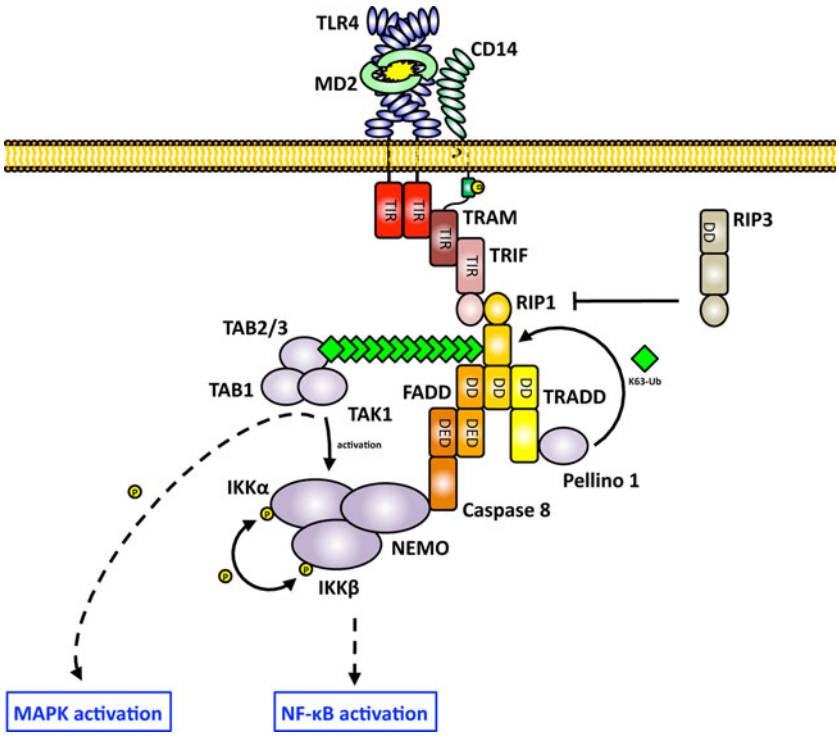

Fig. 3 TRIF-dependent NF-κB/AP-1 activation. TRAM and TRIF are recruited to endosomal TLR4 after TIRAP:MyD88 dissociation from the internalized receptor. Through its RHIM, TRIF mediates direct recruitment of RIP1, which acts as a scaffold for the DD-containing proteins FADD and TRADD. Inactive RIP3 negatively regulates this process by competing with RIP1 for binding TRIF. TRADD binds Pellino1, an E3 ligase that catalyzes K63-linked polyubiquitination of RIP1. NF-κB/AP-1 activation is operated through the concomitant recruitment to the receptor complex of TAK1, which interacts with modified RIP1 via TAB 2/3, and IKKs. IKK recruitment is thought to occur by means of uncleaved caspase 8, which bridges FADD and NEMO. TAK1 in turn mediates IKK activation independently of its kinase activity, which is instead required for downstream MAPK activation

caspase 8 axis is a well-established regulatory system for the initiation of death receptor-induced extrinsic apoptosis. According to this paradigm, FADD and the inactive pro-caspase 8 are part of a death-induced signaling complex (DISC) that drives pro-caspase 8 oligomerization and its "proximity-induced self activation" [152]. Activated, self-cleaved caspase 8 functions as an initiator caspase that triggers the catalytic activity of downstream executioners caspases, which in turn cleave a large panel of substrates, leading to apoptotic cell death. Interestingly, FADD and caspase 8 have been shown to mediate the pro-apoptotic effect of TRIF overexpression, a cellular process that relies on RIP1 recruitment by TRIF [153]. The TRIF-mediated, caspase 8-dependent, pathway has also been reported to contribute to the early apoptosis of bacterially infected innate immune cells [154] through a mechanism that is distinct, but not mutually exclusive, from the DC-specific CD14-NFAT pathway described below [41]. However, several studies have shown that, in addition to its pro-apoptotic effect, caspase 8 can contribute to NF-κB activation upon overexpression [155] or stimulation of TRIF-dependent TLRs. Activation of NF-κB by TLR3 and TLR4, but not TLR9, is significantly impaired in caspase 8-deficient B cells due to a delayed nuclear translocation of the transcription factor [156, 157]. Additionally, both caspase 8 and FADD have been involved in TLR3- and TLR4-induced proliferative B cell response, suggesting that they act together upon TLR stimulation [157]. How caspase 8 participates in NF-κB signaling is unclear, but it is believed that caspase 8 acts as a scaffold to recruit the IKK complex independently of its protease activity [155, 157]. Nevertheless, these observations can be synthesized into a model of TRIF-dependent NF-κB activation that relies on RIP1 binding to TRIF (Fig. 3). The DD of RIP1 then interacts with the DD of FADD, which in turn recruits caspase 8 via a death-effector domain (DED). Caspase 8 brings the IKK to TAK1, whose recruitment to the signaling complex is dependent on RIP1 K63-linked polyubiquitination (see above). It has to be noted that TRADD is also recruited to RIP1 and that it can potentially bind FADD via a DD–DD interaction. Therefore, it remains to be determined whether TRADD participates in FADD–caspase 8 recruitment downstream of RIP1. Although further experimental support will be required to substantiate this model of caspase 8-dependent NF-κB activation, its biological importance is likely to be relevant,

as this pathway also seems to be activated by other, non-TLR, virus-sensing receptors (RIG-I and MDA5) [158].

TRAF3-dependent IRF3/7 activation Type I IFNs are pleiotropic cytokines that regulate critical cellular functions, most notably innate immune antiviral and antibacterial defense. IFN-α and IFN-β are the most representative and biologically relevant family members, as they are released upon viral or bacterial insults to signal auto/paracrine cell activation. Type I IFN production is controlled at the transcriptional level by the IRF family of transcription factors, with IRF3 and IRF7 acting as key regulators of type I IFN gene expression. In resting cells, a conformationally inactive monomer of IRF3 (and IRF7) localizes in the cytosol. Upon receptor stimulation, IRF3 undergoes phosphorylation by a stimulus-activated kinase, followed by homo- or heterodimerization with IRF7. IRF3:IRF3 or IRF3:IRF7 dimers then translocate into the nucleus, where they promote specific gene expression programs [159]. IRF3 and IRF7 are differentially involved in TRIF-dependent signaling, as they are both required for TLR3-mediated type I IFN production, but IRF3 only is activated downstream of TLR4. IRF3 phosphorylation induced by TLR3 and TLR4 is regulated by two IKK-related protein kinases, TBK1 and IKKε [160, 161], with TBK1 being the major contributor to IRF3-dependent type I IFN production [162] (Fig. 4). TBK1 is also the target of negative regulation by the phosphatase SHP2 [163]. Although TBK1 and IKKε share significant sequence and structure homology with IKKα and IKKβ, their role in NF-κB activation is unclear. IRF3 activation by TBK1 and IKKε has been proposed to rely on the upstream adaptors TANK [164], NAP1 [165], and SINTBAD [166]. TANK is a binding partner for several TRAF family members [164] as well as for TBK1 and IKKε [167], and it has been reported to regulate virus- and TLR-induced type I IFN production by bridging TBK1 and IKKε with TRAF3 in vitro [168, 169]. TANK has also been shown to interact with NEMO [170] and to promote the formation of a functional IKK complex that is required for IRF3 activation by RIG-I [171]. Also NAP1 and SINTBAD, which share several structural features with TANK, have consistently been shown to be required for IRF3 phosphorylation [166, 172], likely playing nonredundant roles in bridging TBK1 and IKKε to upstream activators. IRF3 activation by TLR3 or TLR4 is strictly dependent on the recruitment to TRIF of the adaptor TRAF3 [120, 128], a protein whose additional role in limiting MyD88-dependent MAPK activation has already been described in this review. The function of TRAF3 in TLR-induced IRF3 activation is to recruit TBK1 and IKKε into the receptor complex as a consequence of the interaction of TRAF3 with TANK (or a TANK-related adaptor). Once at the receptor, the resultant oligomerization of TBK1 and IKKε mediated by TRAF3 probably triggers their own kinase activity through a previous *trans*-autophosphorylating event. However, the molecular mechanism by which TRAF3 exerts its task is the subject of much current research, since TRAF3 also retains an E3 ubiquitin ligase activity that is essential for proper IRF3 activation (Fig. 4). Upon receptor-induced clustering, TRAF3 promotes its own K63-linked polyubiquitination through a process that only partially involves Ubc13 as the E2 enzyme [130]. An additional contribution to TRAF3 polyubiquitination is likely to come from Ubc5, which a recent study identified as the major E2 in RIG-I/MDA5 antiviral signaling [173]. The discovery of DUBA as a negative regulator of K63-linked polyubiquitination of TRAF3 provides further evidence of this post-translational modification in TRIF signaling to IRF3 [174]. Similarly to TRAF6 in MyD88-dependent signaling, K63-linked polyubiquitination of TRAF3 is believed to promote the recruitment of ubiquitin-binding proteins. One such protein is NEMO, which has been shown to interact in vitro with TBK1 and IKKε through TANK [170] and to be essential for RIG-I-induced IRF3 activation [171] through its ubiquitin-binding ability [173]. Notably, the K63-linked polyubiquitination of TANK has also been reported [169], suggesting that TRAF3- and/or TANK K63-linked polyubiquitination triggers TBK1 and IKKε recruitment through binding NEMO, which in turn interacts with TANK. Nevertheless, this mechanical model cannot be considered to be consistent at the present time since several points are still obscure or contradictory. First of all, no contribution of NEMO for IRF3 activation by TLR3 or TLR4 has yet been reported; rather, all data have been generated using RIG-I as the activating receptor. Additionally, the generation of TANK-deficient mice has revealed that TANK is not involved in type I IFN response but that it acts as a negative regulator of TLR-induced proinflammatory cytokine production, probably through suppression of K63-linked polyubiquitination of TRAF6 [175]. The observation that TANK is dispensable for IRF3 activation in vivo [175] raises the question of whether NAP1 or SINTBAD may operate this function by binding NEMO. Another possibility is that TBK1 itself recruits NEMO, as TBK1 has recently been shown to be the target of K63-linked polyubiquitination by the E3 ligase Nrdp1 [176]. Further clarification of these points will result in reliable knowledge on how IRF3 is mechanically activated by K63-linked polyubiquitination of TRAF3 following TRIF stimulation. All type I IFN-inducing TLRs trigger IRF activation from an endosomal location, a generalization that includes the TRIF-dependent TLR4 signaling pathway. The functional specialization of receptors is therefore underlined by spatial separation, even though the molecular determinants that mediate site-specific signal transduction are still not perfectly clear. TRAF3

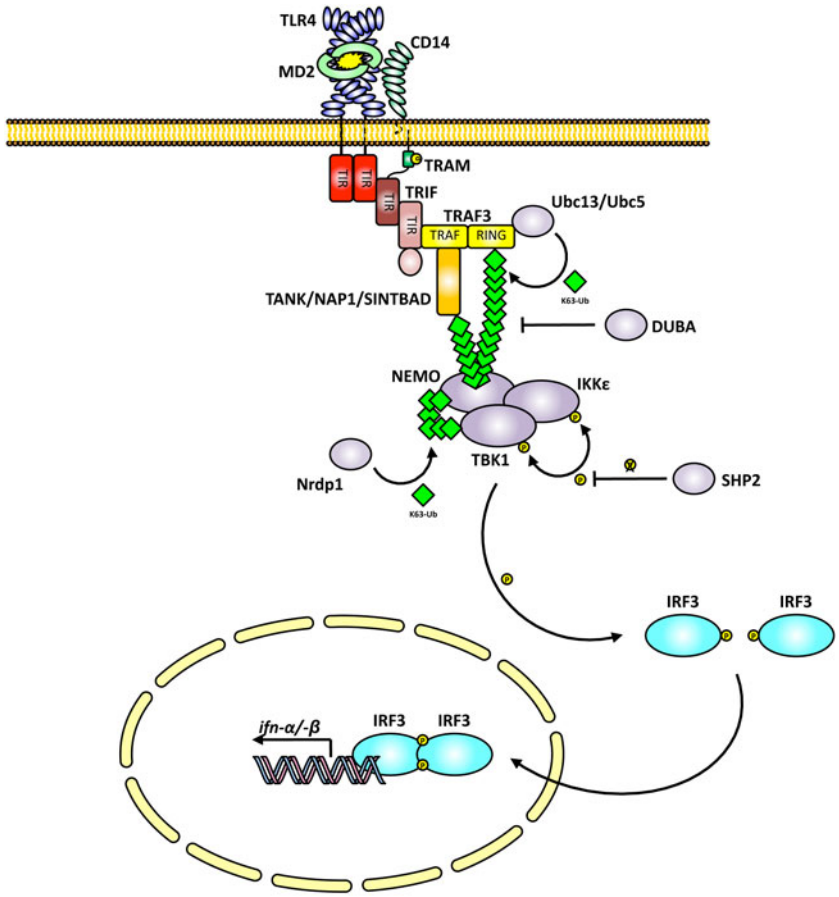

Fig. 4 TRIF-dependent IRF3 activation. TRIF mediates direct recruitment of TRAF3 to endosomal TLR4 and promotes its consequent oligomerization, resulting in the Ubc13/Ubc5-dependent K63-linked polyubiquitination of TRAF3 and possibly of the downstream adaptors TANK/NAP1/SINTBAD. K63-linked polyubiquitination of TRAF3 is negatively regulated by the deubiquitinase DUBA. TRAF3 and/or one of the TRAF3-interacting adaptors recruit TBK1 and IKKε via the ubiquitin-binding domain of NEMO, thereby linking upstream signaling with IRF3 activation. The interaction of TBK1 with NEMO is also favored by the Nrdp1-dependent K63-linked polyubiquitination of TBK1. Upon transautophosphorylation, TBK1/IKKε are activated and phosphorylate IRF3 monomers, which in turn dimerize and translocate into the nucleus to promote type I IFN gene expression

is thought to discriminate between plasma membrane and endosomal signaling, since it is shared by all intracellular (including the TRIF branch of TLR4), but not cell membrane TLRs (including the MyD88 branch of TLR4). TRAF3 has been reported to localize in pleiomorphic cytosolic structures, a subcellular distribution that precludes its interaction with MyD88 at the cell surface [74]. Artificial targeting of TRAF3 to the plasma membrane allows TLR2 to induce type I IFN expression, suggesting that TRAF3 localization per se regulates the ability of endosomal TLRs to activate IRFs [74]. However, it has to be noted that TRAF3 localization might not be the only discriminating element, since TRAF3 can be recruited at the plasma membrane by some receptors [74], including TLR4 itself [130]. As described above, TRAF3 efficiently interacts with MyD88 upon LPS stimulation where it prevents cytosolic MAPK activation until it gets degraded as a consequence of K48-linked polyubiquitination by the E3 enzymes cIAP1/2 [130] (Fig. 2b). After TLR4 endocytosis, TRAF3 is recruited to TRIF, where it is K63-linked polyubiqutinated by Ubc13 (and possibly Ubc5) to trigger NF-κB activation [130] (Fig. 4). Therefore, it appears that the localized differential functionalization of TRAF3 through K48- or K63-linked polyubiquitination, rather than its simple subcellular distribution, is responsible for inducing IRF activation from endosomal locations. A careful investigation will be required to validate this model for other TLRs as well and to unveil the mechanisms regulating the selective localization (or activation) of specific ubiquitinating enzymes (Fig. 4).

CD14: a co-receptor with crucial signaling functions

To this point in this review we have described the known functions of CD14 as a sensing receptor for TLR4 as well as other TLRs. However, we have recently described a novel signal transduction pathway induced by LPS that exclusively relies on CD14 for activation of the transcription factor NFAT in DCs [41]. These findings provide the definitive demonstration that CD14 can act independently of TLRs as a transducing receptor (Fig. 5). DCs retain the ability to release IL-2 upon stimulation with several pattern recognition receptors, including TLR4 [177, 178], a key event for natural killer (NK) cell activation [179]. Since IL-2 expression in T lymphocytes is regulated at the transcriptional level by NFAT [180], we undertook a study to assess whether LPS stimulation in DCs is also able to induce activation of this transcription factor. As it turned out, CD14 is responsible for the induction of a rapid and

transient influx of Ca^{++} ions in LPS-stimulated DCs. The consequent increase in the cytosolic Ca^{++} concentration ($[Ca^{++}]_I$) triggers the activation of calcineurin, a phosphatase that removes phosphate groups from cytosolic, inactive NFAT, thereby promoting its nuclear translocation. Strikingly, experiments in DCs doubly deficient in TLR4 and TLR2 as well as MyD88 and TRIF clearly reveal that activation of NFAT by LPS in DCs occurs with no contribution at all from TLR4 or any other TLR. Instead, CD14 is necessary for inducing Ca^{++} influx and NFAT activation after LPS stimulation in DCs. The downstream effectors of this CD14-dependent pathway have also been identified, with Src family kinases (SFKs) and PLCγ2 playing essential roles in triggering Ca^{++} influx and NFAT activation. These and other data, in addition to previous knowledge, allow a reliable model for CD14-dependent signal transduction in DCs to be constructed whereby LPS stimulation triggers the clustering of CD14 molecules in lipid rafts (Fig. 5). Through an ill-defined mechanism, CD14 clustering results in SFK activation (possibly Lyn), which in turn activates PLCγ2 by phosphorylation. PLCγ2 acts by hydrolyzing its substrate $PI(4,5)P_2$ into the second messengers, $I(3,4,5)P_3$ and diacylglycerol (DAG). Whereas DAG probably signals NF-κB activation through PKCs (protein kinase C), $I(3,4,5)P_3$ triggers the opening of $I(3,4,5)P_3$-regulated ion channel receptors on the cell surface, resulting in a single wave of extracellular Ca^{++} influx that ultimately promotes calcineurin activation, NFAT dephosphorylation, and nuclear translocation. Apart from describing a novel signaling ability of CD14, this model raises a number of interesting questions about the regulation of this pathway at the molecular level. For example, how does CD14 transduce the LPS recognition signal intracellularly? In this regard, it has to be noted that CD14 appears to signal NFAT activation through its GPI-anchor, rather than by presenting LPS to an unknown transmembrane protein other that TLR4. Indeed, soluble CD14 (sCD14) restores sensitivity to low doses of LPS in CD14-deficient DCs in terms of proinflammatory cytokine production, but not in terms of Ca^{++}-NFAT signaling. Furthermore, disruption of lipid raft integrity with a cholesterol-depleting agent abolishes the ability of wild-type DCs to induce a Ca^{++} response to LPS. These observations strongly support the hypothesis that membrane-anchored CD14 that resides in lipid rafts directly promotes NFAT activation, although we can not formally exclude the involvement of additional transmembrane players. We propose that the clustering of CD14 induced by LPS promotes the aggregation and consequent activation of other lipid raft-associated signaling proteins, namely SFKs, which in turn activate PLCγ2. Consistent with this hypothesis is the recent finding that colloidal gold-induced cross-linking of the GPI-anchored receptor CD59 results in the transient lateral immobilization in lipid rafts of Lyn and Gα2, which triggers the activation of Lyn and, consequently, PLCγ2 [38, 39]. An additional feature of CD14-dependent signaling to NFAT that merits further investigation is the mechanism by which an increase of $[Ca^{++}]_I$ is obtained upon LPS stimulation in DCs. T cell receptor-induced calcineurin activation is typically operated through a two-step Ca^{++} mobilization system called store-operated Ca^{++} entry (SOCE). According to the SOCE paradigm, $I(3,4,5)P_3$ promotes the opening of specific ion channel receptors (IP_3-R) localized on the endoplasmic reticulum (ER). Since the $[Ca^{++}]$ in the ER is higher than that in the cytosol, this results in a transient wave of Ca^{++} mobilization from the ER. The consequent depletion of the Ca^{++} store is then sensed and communicated to the plasma membrane by means of the STIM-Orai1 system, where additional ion channels allow a second, sustained wave of Ca^{++} influx. Interestingly, LPS induces a single and transient influx of extracellular Ca^{++}, with no contribution from intracellular Ca^{++} stores, which is still sufficient to activate NFAT. This suggests that LPS-induced Ca^{++} signaling in DCs does not rely on a classical SOCE mechanism, but that $I(3,4,5)P_3$ may trigger direct activation of functional plasma membrane IP_3-Rs, as has already been observed in B cells [181]. The CD14-dependent NFAT activation induced by LPS plays a crucial function in regulating the life cycle of activated DCs, which undergo apoptosis shortly after stimulation. Stimulus-induced apoptosis of DCs represents a strategy to limit T cell activation in lymph nodes and to prevent an excessive immune response. We have shown that, upon DC stimulation with LPS, activated NFAT proteins (namely NFATc2 and NFATc3) promote the expression of several genes with a pro-apoptotic function that altogether induce cell death. Among these genes, we identified *Nur77* as an NFAT-dependent regulator of DC apoptosis. It has to be noted that although NFAT activation is normally observed in TLR4-deficient DCs after LPS treatment, no appreciable gene expression occurs in these conditions, suggesting that cooperation with NF-κB and AP-1 is a pre-requisite for NFAT to exert its biological function (Fig. 5). We also found that macrophages, which do not undergo LPS-induced apoptosis, do not show a Ca^{++} response or activation of NFAT upon LPS stimulation. However, pharmacological activation of NFAT is sufficient to induce the cell death of macrophages upon LPS treatment, further supporting a role for NFAT as a master regulator of the cell life cycle. Although we still do not know the point where the CD14-dependent pathway is blocked in macrophages, this differential signaling ability between DCs and macrophages provides a molecular explanation for their peculiar life cycle, which is in turn responsible for their diverse

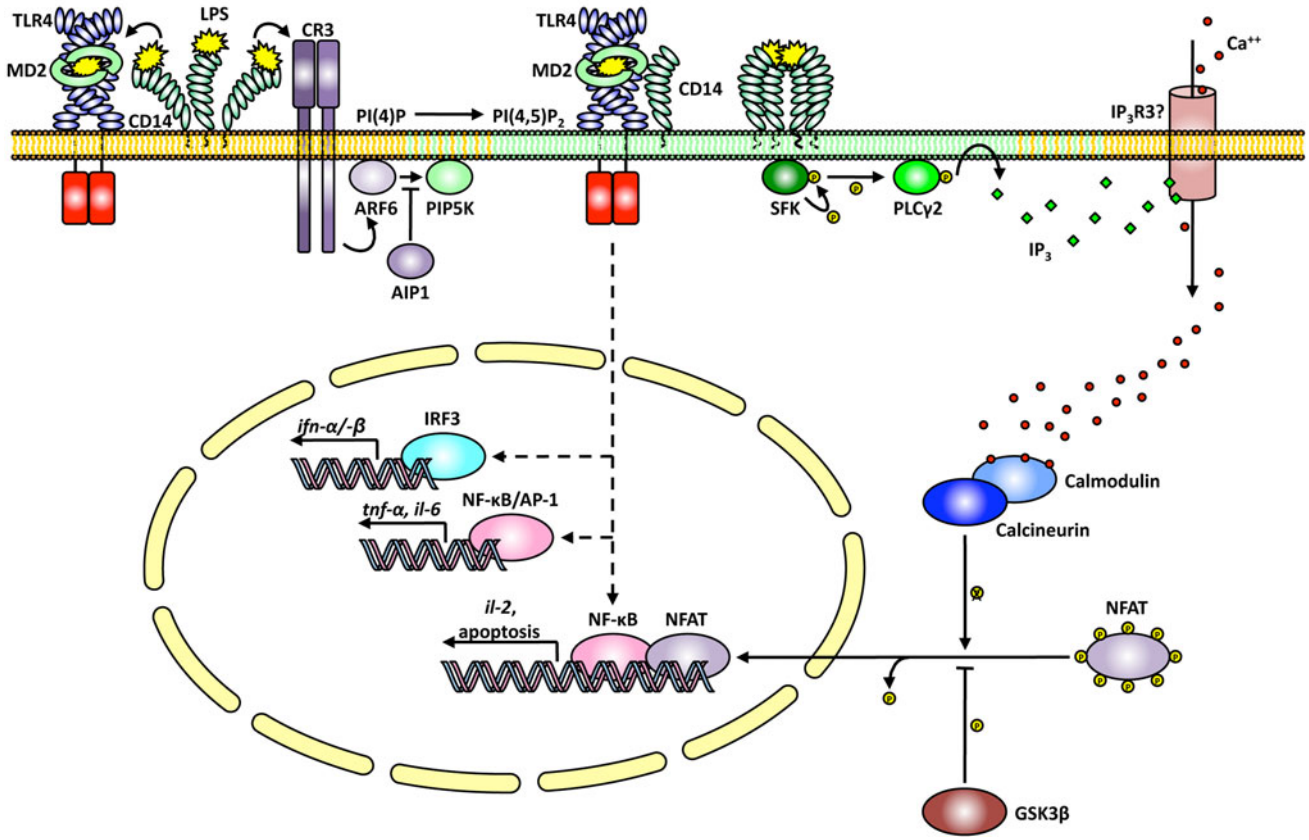

Fig. 5 CD14-dependent and TLR4-independent NFAT activation in dendritic cells. In addition to its role in LPS recognition and presentation to TLR4 and CR3 (see Fig. 1), CD14 has autonomous signaling functions in dendritic cells (DCs). Upon LPS-induced clusterization, CD14 transiently recruits and activates a Src family kinase (*SKF*) member through an ill-defined mechanism that relies on the CD14 GPI anchor and on its residency in lipid rafts. Active SFK then phosphorylates PLCγ2, which in turn catalyzes the hydrolysis of PI(4,5)P2 into the second messengers diacylglycerol (DAG) and IP3. Whereas the biological role of DAG in this system has not been investigated, it is likely to contribute to NF-κB activation through PKCs (not shown). On the other side, IP3 directly triggers Ca^{++} influx by acting on the plasma membrane Ca^{++} channel receptor (IP3R3?). The increased $[Ca^{++}]_I$ stimulates activation of calcineurin, which dephosphorylates NFAT and promotes its nuclear translocation. Active NFAT cooperates with NF-κB to drive the expression of the genes coding for IL-2 as well as several proapoptotic proteins. It has to be noted that, although LPS-induced activation of NFAT in DCs is TLR4 independent, no change in gene expression is observed in the absence of TLR4, which is therefore required for full transcriptional activity of NFAT through activation of NF-κB

biological functions. Therefore, we propose that the relevance of this pathway be carefully investigated in other CD14-expressing cell types in order to elaborate appropriate intervention strategies for the treatment of diseases, such as sepsis, that might involve unphysiological functionalities of CD14.

Conclusions and perspectives

The discovery of mammalian TLRs coincided with a rebirth of the field of innate immunity, which has yielded crucial insights into a multifaceted defense strategy against invading pathogens. TLRs are crucial mediators of this protective mechanism and thus represent valuable targets for the therapeutical boosting of innate immune response needed for vaccine adjuvanticity. On the other side, dysregulated activation of TLRs is causal to a number of pathological conditions in which overt inflammation is observed, i.e., sepsis, autoimmune diseases, and cancer. A careful analysis of existing and novel transgenic mouse models as well as the in-depth biochemical and cell biological investigation of TLR signaling will be therefore required for the generation of an experimental framework to be translated into a useful clinical setting.

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