Closing in on Toll-like receptors and NOD-LRR proteins in inflammatory disorders

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Our immune system faces the intricate task of eliminating pathogenic microorganisms, while autoimmunity against self-components or intestinal flora must be prevented. This complex task demands an exceptional level of regulation and specificity. Hereto, immune cells are equipped with a myriad of surface receptors, including cytokine-receptors and pathogen recognition receptors. In this review we describe two important PRR families, Toll-like receptors and nucleotide-binding oligomerization domain leucine-rich repeat proteins, and their crucial role in instruction of the immune response. Interestingly, these key immune-receptors have recently been identified as major players in several immune disorders, such as systemic lupus erythematosus, Crohn's disease, auto-inflammatory diseases and rheumatoid arthritis. These findings, and their implications for future research and therapy of immune disorders, will be discussed.

Our immune system is well equipped to recognize specific, highly conserved structures unique to microorganisms of a given class. These so-called pathogen-associated molecular patterns (PAMPs) are recognized by pathogen recognition receptors (PRRs), such as C-type lectins [1], members of the nucleotide binding oligomerization domain (NOD) leucine-rich repeat (LRR) family [2] and Toll-like receptors (TLRs) [3]. TLRs owe their name to the *Drosphila* protein Toll. Toll was initially studied for its involvement in embryonic dorsoventral axis formation. However, Toll$^{-/-}$ animals quickly succumbed to massive fungal infection, implying a role for Toll in immunity [4]. Subsequent bioinformatics analyses led to the identification of mammalian TLRs. To date, 13 TLRs have been defined. However, certain TLRs can form heterodimers, which further increases TLR diversity. TLRs are type 1 transmembrane receptors, which recognize their ligands via a conserved C-terminal LRR domain. For some TLRs, ligand binding is dependent on the presence of accessory molecules [5,6]. Many pathogen-derived structures, such as lipopolysaccharide (LPS), bacterial lipoproteins and double-stranded RNA are sensed by distinct TLRs (**Table 1**). In addition, it has been suggested that several endogenous structures – mostly associated with cell death, tissue damage or infection – can activate TLRs, such as heat-shock proteins [7], β-defensins [8] and fibrinogen [9]. However, these findings should be approached prudently, as contamination with, for instance, endotoxins could lead to serious misinterpretation of results [10,11].

All members of the TLR family share a cytoplasmic domain called Toll/interleukin (IL)-1 receptor (TIR) domain, for its homology with the respective region of the IL-1 receptor. Activation of TLRs induces the recruitment of adaptor proteins that serve as a scaffold for downstream signaling molecules. The adapter protein myeloid differentiation factor (MyD)88 is involved in signaling of most TLRs, with the exception of TLR3, which signals via Toll/IL-1 receptor domain-containing adapter-inducing interferon IFN-β (TRIF). The use of different adaptor proteins – and thus activation of distinct signaling pathways – brings specificity to TLRs and directs the type and magnitude of the immune response. While TRIF effectively activates interferon regulatory factor (IRF)-3, resulting in interferon (IFN)-β production [12,13], MyD88 mediates transcription of cytokines and chemokines via nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathways. Interestingly, the recently discovered adapter proteins MyD88 adapter-like (Mal) [14] and TRIF-related adapter molecule (TRAM) [15] add to the further diversification of the TLR signaling network. To avoid excessive inflammation or autoimmunity following TLR activation, feedback mechanisms are of paramount importance. Indeed, a plethora of regulatory molecules and circuits are in place to control TLR responses. For instance, single immunoglobulin IL-1 receptor-related protein (SIGIRR) attenuates the recruitment of receptor-proximal signalling components [16], while stromal (ST)2 cells can prevent the translocation of NF-κB to the nucleus [17].

Keywords: anakinra, auto-inflammatory disease, dendritic cells, NOD-LRR protein, rheumatoid arthritis, Toll-like receptor

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Table 1. Overview of Toll-like receptor ligands and sources.

TLR	Ligand	Source	Ref.
TLR1 and TLR2	Triacyl lipopeptides*	Bacteria	
	Lipoarabinomannan*	Mycobacteria	
	PorB porin*	*Neisseria meningitidis*	
	Nystatin	Anti-fungal drug (*Streptomyces noursei)*	[121]
TLR2	Lipopeptide/lipoprotein*	Bacteria	
	Lipoteichoic acid*	Gram-positive bacteria	
	Lipopolysaccharide*	*Plasmodium gingivalis, Listeria interrogans*	
	Lysophosphatidylserine*	*Streptomyces mansoni*	
	HSP60*, HSP70*, HSPB8	Host, *Helicobacter pylori*	[122]
	HMGB1*	Host	
	Biglycan	Host	[123]
TLR2 and TLR6	Diacyl lipopeptides*	*Mycoplasma*	
	Lipoteichoic acid*	*Staphylococci, Streptococci*	
	GPI*	*Plasmodium falsiparum*	
TLR3	dsRNA*	West-Nile virus, Cytomegalovirus, *S. mansoni*	
	siRNA*	Synthetic	
	mRNA*	Host	
TLR4	Lipopolysaccharide*	Gram-negative bacteria	
	Taxol*	plants	
	Anthrolysin O*	*Baccilus anthracis*	
	Phosphorylcholine*	Filarial nematode	
	HSP60*, HSP70*	*Chlamydia pneumoniae,* host	
	β-defensin 2*	Host	
	Fibrinogen*	Host	
	Hyaluronic acid*	Host	
	Fatty acids*	Host	
	Modified LDL*	Host	
	Biglycan	Host	[123]
	Levan	Host	[124]
	Heparan sulfate	Host	[125]
TLR5	Flagellin*	Bacteria	
TLR7	ssRNA*	Influenza, HIV-1, parechovirus 1	
	Imidazoquinoline*	Synthetic	
	Loxoribine*	Synthetic	
TLR8	ssRNA*	Coxsackie B virus, parechovirus 1	
	Imidazoquinoline*	Synthetic	
TLR9	CpG DNA*	Bacteria, synthetic, DNA viruses	
	Hemozoin*	*Plasmodium falsiparum*	
	Chromatin–IgG complexes*	Host	
TLR10	Not determined*		
TLR11	Profilin-like molecule*	*Toxoplasma gondii*	

**Adapted from Isshi* et al. *[126], supplemented with most recent data from literature.*
dsRNA: Double-stranded RNA; GPI: Glycosyl-phosphatidylinositol; HMBG: High-mobility group box; HSP: Heat-shock protein; LDL: Low-density lipoprotein; siRNA: Short interfering RNA; ssRNA: Single stranded RNA; TLR: Toll-like receptor.

Finally, the MyD88 splice-variant MyD88s can serve as a 'decoy' adaptor molecule that prevents the recruitment of IL-1 receptor-associated kinase (IRAK)-4 and subsequent downstream signaling [18].

In conclusion, TLRs play a central role in the response to danger signals, such as the presence of microorganisms or tissue damage. Triggering of TLRs leads to activation of highly complex and intertwined signaling pathways that control immunity.

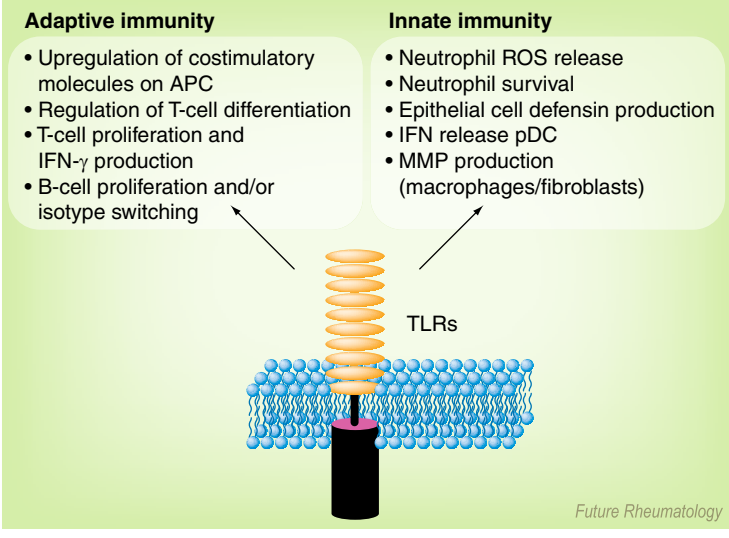
Toll-like receptor expression & location

Different combinations of TLRs and TLR signaling molecules are expressed in various cell types involved in the induction of innate immune responses. Monocytes, macrophages and neutrophils are all equipped with their own subset of TLRs, enabling them to execute their effector function. However, TLR expression is not limited to innate immune cells, as they can also activate T and B cells, and nonimmune cells located at the interface of the interior and exterior milieu (**Figure 1**). For example, airway epithelial cells produce inflammatory cytokines and antimicrobial peptides in response to PAMPs or pathogens in a TLR-dependent manner [19–21].

The expression of TLRs is dynamic and can be increased by proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IFN-γ and IL-6, which contribute to a higher sensitivity for TLR ligands and amplification of the inflammatory response [22–24]. On the other hand, TLR protein expression is tightly controlled via ubiquitin-mediated protein degradation [25]. Furthermore, prolonged exposure to microbial components results in hyporesponsiveness, as a result of decreased TLR expression and the upregulation of endogenous inhibitory molecules; a mechanism to prevent disproportionate inflammation [26]. TLRs also differ in their location within a cell. Whereas TLR1, -2, -4, -5 and -6 are present on the plasma membrane, TLR3, -7, -8 and -9 reside mainly within the endosomal/lysosomal compartment. The differential subcellular location of TLRs relates to the nature of the ligands that are recognized. Thus, TLRs at the cell surface mainly respond to extracellular pathogens, while intracellular TLRs are triggered by nucleic acids from bacteria and viruses, mostly following uptake of pathogens or pathogen-infected cells. In addition, the intracellular localization avoids unwanted, and potentially harmful, inflammatory responses against extracellular self-DNA or mRNA released from dying or dead cells. Likewise, the polarized expression of TLR5 in intestinal epithelial cells prevents inflammatory responses to commensal bacteria in the intestine. TLR5 is absent on the luminal side of the cell and is only triggered by its ligand, flaggelin (the PAMP in flagella from motile bacteria), once the epithelial barrier is breached by pathogenic bacteria [27,28].

In summary, TLR expression in various tissues and cell types is dynamic and can be increased by several proinflammatory cytokines. Tissue- or cell-type-specific localization of TLRs is organized such that instant recognition of invading pathogens is ensured, but responses to self-antigens are avoided.

Figure 1. Effect of TLR triggering on innate and adaptive immune responses.



APC: Antigen-presenting cell; DC: Dendritic cell; IFN: Interferon; MMP: Matrix metalloprotease; ROS: Reactive oxygen species; TLR: Toll-like receptor.

Role of Toll-like receptors in the adaptive immune response

Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system, and direct the type and course of an immune response. DCs can initiate immune responses against pathogens or tumors, but also prevent autoimmune responses that are harmful to the host. Immature DCs are present in virtually all organs and tissue,s and they continuously sample their environment for the presence of microorganisms. Captured antigens are processed into peptides and subsequently presented to lymphocytes in lymph nodes. DCs can also activate

natural killer cells and produce IFNs, thus linking the innate and adaptive immune system [29]. DCs express a broad repertoire of TLRs, and activation of these receptors results in upregulation of major histocompatibility complex (MHC) and costimulatory molecules, and migration of DCs to the secondary lymphoid organs, where they activate naive T cells. The nature of the signals that DCs receive, especially through TLRs and cytokine receptors, determines the type of immune response induced. Unstimulated DCs or DCs receiving immune-inhibitory signals induce immune tolerance. On the other hand, triggering of TLR4 on DC results in T-helper (Th)1 polarization, while TLR2 activation rather supports a Th2 response [30,31]. These findings have significantly contributed to our understanding of the classical Th1–Th2 paradigm, and indicate that the combination of TLRs activated upon pathogenic encounter plays a pivotal role in directing the type of immune response against that particular pathogen [32]. However, this phenomenon can sometimes be exploited by microorganisms. For example, *Candida albicans* is shown to induce Th2 or Treg differentiation via production of cytokines, such as IL-10, thereby preventing the Th1 response necessary for its eradication [33].

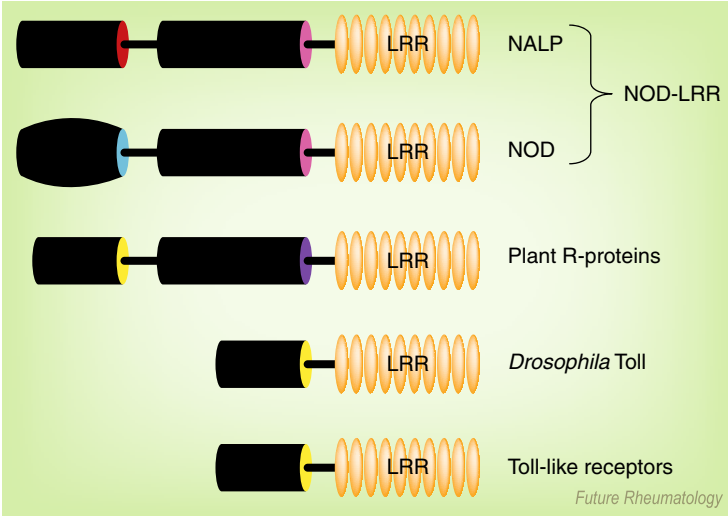
NOD-LRR protein family

Recently, new LRR-containing PRRs have been identified and collectively christened the NOD-LRR family [2]. NOD-LRRs have a high structural homology to plant R proteins (**Figure 2**), which are involved in innate immune defense [34]. NOD-LRRs reside in the cytosol and are thought to be involved in sensing the presence of intracellular microorganisms. All NOD-LRR proteins share a C-terminal LRR domain, a central NOD domain – used to form dimers or oligomere complexes – and a distinct N-terminal effector domain. The NOD-LRR family can be divided into four distinct subfamilies: the CIITA, interleukin 1 β-conveting enzyme (ICE)-protease activating factor (IPAF), NOD and NALP subfamilies, of which the latter two will be discussed further.

NOD1 and 2 respond to distinct moieties of the bacterial cell wall component peptidoglycan [35–38]. NODs are expressed in monocytes, macrophages and DCs, but also in nonhematopoietic cell types, such as intestinal epithelial cells. Triggering of NODs results in homodimerization, the recruitment of adapter protein receptor-interacting protein (RIP)-like interacting CLARP kinase (RICK) via homotypic caspase recruitment domain (CARD)–CARD interactions and induction of subsequent signaling events [39]. Triggering of endogenous NODs leads to only a moderate secretion of cytokines through activation of NFκB and mitogen-activated protein kinases (MAPKs). However, recent data indicate that NOD stimulation strongly synergizes with TLR triggering for the production of both pro- and anti-inflammatory cytokines [40,41].

Another NOD-LRR subfamily is the NALP family, consisting of 14 proteins that share structural similarity with the NODs [42]. Rather than an N-terminal CARD domain, NALPs display a so-called pyrin domain. Certain NALPs, especially NALP3 (cryopyrin/CIAS1/PYPAF), have recently been discovered to be key regulators of the inflammatory cytokine IL-1β [43]. Several NF-κB-activating signals induce production of the inactive pro-IL-1β protein. The processing of pro-1β into active IL-1β is mediated by activation of a multiprotein complex referred to as the inflammasome, of which NALPs and pro-inflammatory caspases are crucial components [44]. This activation of the inflammasome can occur independently of TLRs. Binding of NALPs to apoptosis-associated speck-like protein (ASP) containing a CARD leads to recruitment and activation of caspase-1 (also known ICE) and production of bioactive IL-1β. Since NALPs posses LRR domains similar to that of TLRs, they are thought to play a role in pathogen recognition. Recent studies by the Tschopp and Núnêz groups identified several compounds that can trigger NALP pathways, such as the NOD2 activator muramyl dipeptide (MDP) [45], bacterial RNA, imidazoquinolines (which also activate TLR7/8) [46] and gout-associated uric acid crystals [47]. However, it remains to be determined whether these structures directly bind to and trigger NALPs, or whether associated factors are required for their activation. Future research will undoubtedly lead to the identification of other NALP ligands, similar to the ever-growing list of TLR ligands that has been discovered in recent years. In parallel with regulation of TLR signaling, the presence of endogenous inhibitors, such as pseudo-ICE, inhibitor of pro-caspase-1 activation (ICEBERG) [48] and proteinase inhibitor (PI-9) [49], which interfere with IL-1β processing, is crucial to prevent disproportionate inflammatory responses.

Figure 2. Structural organization of the NOD-LRR family members, as well as their homologs, the plant R proteins.



Both mammalian and plant proteins possess a C-terminal ligand-sensing LRR domain, a central oligomerization domain (called NBARC in R proteins) and an N-terminal effector domain. Likewise, *Drosophila* Toll and mammalian Toll-like receptors contain a C-terminal LRR-domain and an N-terminal TIR effector domain, which binds intracellular adapter proteins (e.g., MyD88), initiating signaling.
CARD: Caspase recruitment domain; LRR: Leucine-rich repeat; NBARC: ATPase domain; NOD: Nucleotide oligomerization domain; TIR: Toll-interleukin-1 receptor.

Toll-like receptors & NOD-LRR in immune disorders

Systemic lupus erythematosus

Since TLRs are such potent inducers of both innate and adaptive immune responses, they are implicated in many immune disorders, such as Crohn's disease (CD) [50,51] and Type 1 diabetes mellitus [52]. The clearest example is the relationship between TLR9 and the autoimmune disease systemic lupus erythematosus (SLE). SLE is thought to be related to defective clearing of apoptotic cells and characterized by chronic inflammation initiated by deposition of immune complexes (IC) in target organs [53]. The ICs consist of antibody/DNA and antibody/nucleoprotein complexes, and they cause the release of a variety of chemokines and cytokines, thereby attracting, for instance, neutrophils and DCs. *In vitro* stimulation with serum or purified DNA-IC from SLE patients results in significant IFN-α production by a rare subpopulation of DCs, referred to as plasmacytoid (p)DCs [54]. It has now been determined that IFN-α release by pDC is induced in a TLR9 and FcR-dependent manner [55]. It was found that DNAICs are taken up via FcγRIIa and translocate to acidic lysosomes, where binding of DNA to TLR9 triggers the production of IFN-α. Antibodies against FcγRIIa or addition of chloroquine, a known inhibitor of TLR9 signaling, both efficiently blocked IFN-α production following stimulation of pDC with DNA-ICs [56]. An excellent review dealing with the complex interplay of these receptors in SLE has recently been published [54]. The finding that polymorphisms in FcγRIIa are correlated with susceptibility to SLE further supports a role for this receptor in the delivery of complexes into the cell. To date, an association between TLR9 polymorphisms and susceptibility to SLE has not been established [57]. Instead, it has been found that polymorphisms in TLR5 are associated with protection from the development of this disease [58], via a mechanism that is currently unknown.

These data indicate that cooperation between seemingly distinct receptors, including TLRs, contributes to the perpetuation of the inflammatory response against DNA and/or nuclear proteins that is characteristic of SLE.

Crohn's disease

CD is a chronic Th1-mediated inflammatory disease that can affect any part of the gastrointestinal tract. CD is a multifactorial disease, with a strong genetic component and a role for environmental factors. It is generally believed that the disease results from an exaggerated immune response directed against the normal intestinal flora. This idea is supported by the finding that animals kept in a germ-free environment generally do not develop colitis [59]. The vast number of bacteria in the gut represents a plethora of potential PRR-activating structures, and this situation demands a tight immune regulation. Both TLRs and NOD-LRR proteins play an important role in intestinal inflammatory responses. In the mouse colitis model of dextran sulfate sodium (DSS), TLR9 stimulation with DNA derived from luminal bacteria results in elevated pro-inflammatory cytokine and chemokine production, and more pronounced histopathological damage in the colonic mucosa in wild-type mice, but not TLR9$^{-/-}$ mice [60], indicating that TLR9 signaling can contribute to intestinal inflammation. Similar responses were obtained after TLR5 activation using flagellin [27]. Interestingly, flagellin is not only recognized by TLR5. Cytosolic flagellin can activate IPAF, a NOD-LRR protein, which results in production of IL-1β [61,62]. The availability of two sensory

pathways for flagellin potentially enables regulation of the intensity of the immune response, depending on the virulence of the pathogen encountered. Interestingly, TLR2 agonists do not cause an inflammatory response in the DSS colitis model [27], an effect that might be related to the induction of a Th2 cytokine profile following TLR2-triggering. Despite the fact that MyD88-dependent TLR5 and TLR9 responses can contribute to colonic inflammation, it was recently found that MyD88-deficient mice exhibit an increased susceptibility to DSS-induced colitis [63]. This would suggest a protective role for MyD88-dependent signaling in preventing colonic inflammation.

Additonally, on a genetic level, TLRs and NOD2 have been linked to CD. For instance, the TLR4 Asp299Gly polymorphism, which is associated with increased susceptibility to Gram-negative infections, is found more frequently in CD patients than in the healthy population [50,51]. However, the most compelling evidence is found for polymorphisms in NOD2. The 3020insC frameshift mutation in NOD2 that results in a truncated form of the protein is highly associated with CD [64,65]. Although several mouse models have suggested that this mutation results in enhanced NF-κB activation and IL-12 or IL-1β production [66,67], virtually all studies performed with human cells suggest a loss-of-function mutation, resulting in defective recognition of MDP and loss of synergy between TLRs and NOD2 [41,68,69]. In addition, NOD2 knockout (KO) mice were found to have decreased expression of a subgroup of antimicrobial peptides [70]. How exactly a loss-of-function mutation in NOD2 leads to an unwarranted immune response towards intestinal flora is not currently completely clear, but might be related to defective immune regulation by impaired release of immunosuppressive cytokines, such as IL-10 [41,68,69]. Besides its involvement in CD, NOD2 was recently identified as the susceptibility gene for another granulomatous disorder, termed Blau syndrome (BS) [71]. BS is a rare disease that features early-onset granulomatous arthritis, uveitis, skin rash and camptodactyly (**Table 2**). These data indicate that different mutations in NOD2 can have distinct physiological consequences. In the case of BS, the NOD2 mutations all affect the NOD domain of the protein [71,72]. Since the NOD domain is not reported to be involved in the recognition of PAMPs, these mutations may cause ligand-independent NOD2 activation and inflammation, which is also observed in other so-called autoinflammatory disorders, which are discussed below.

NALPs & autoinflammatory syndromes

Autoinflammatory diseases are characterized by recurrent episodes of seemingly unprovoked systemic inflammation that, unlike autoimmune disease, lack high-titer antibodies or the involvement of antigen-specific T cells. Mutations in NALP3 have unquestionably been linked to three prototypical autoinflammatory syndromes: familial cold autoinflammatory syndrome (FCAS) [73], Muckle–Wells syndrome

Table 2. Overview of members of the NOD-LRR family involved in systemic auto-inflammatory disorders with rheumatic manifestations.

Disease	Symptoms	Affected gene	Ref.
MWS	Episodes of rash, arthralgia, fever, conjuctivitis Frequent sensorineural hearing loss	*NALP3*	[73]
FCAS	Cold-induced episodes of rash, arthralgia, fever Conjuctivitis	*NALP3*	[73]
NOMID	Rash, papilledema, uveitis, hepatosplenomegaly Sensorineural hearing loss, arthropathy Epiphyseal boneformation	*NALP3*	[74,75]
Blau syndrome	Granulomatous papular rash, uveitis, iridocyclitis Granulomatous arthritis, camptodactyly	*NOD2*	[71,72]

FCAS: Familial cold autoinflammatory syndrome; LRR: Leucine rich repeat; MWS: Muckle–Wells syndrome; NOD: Nucleotide-binding oligomerization domain; NOMID: Neonatal-onset multisystem inflammatory disease.

(MWS) [73] and neonatal-onset multisystem inflammatory disease (NOMID) [74,75]. These syndromes are now considered to be a continuum of one disease, referred to as cryopyrin-associated periodic syndromes (CAPS). The CAPS share features, such as episodes of fever, arthritis and increased levels of inflammatory markers (**Table 2**). Most NALP3 mutations affect the NOD domain of the protein and it has been hypothesized that they interfere with auto-inhibitory LRR–NOD interaction and cause NALP3 inflammasome activation and IL-1β production – even in the absence of NALP3-activating structures [43]. In line with this, macrophages from MWS patients were found to spontaneously secrete IL-1β [43]. The role of IL-1β in these diseases is further supported by the finding that treatment with the IL1-R antagonist anakinra results in a dramatic improvement in the clinical symptoms and laboratory markers of inflammation in patients with MWS, FCAS and NOMID [76–78].

Toll-like receptors, NOD-LRRs & their involvement in joint inflammation

Rheumatoid arthritis

RA is a common autoimmune disease characterized by inflammation of the synovial joints, leading to cartilage degradation and bone destruction, affecting approximately 1% of the worldwide population. The first evidence that suggested RA to be an autoimmune condition originated from the association of RA with certain human leukocyte antigen (HLA) subtypes [79]. Secondly, various autoantigens, including rheumatoid factor and citrullinated peptides, were found to be associated with susceptibility to, and severity of, RA [80,81]. Despite multiple efforts, the precise role of HLA subtypes and autoantigens in RA pathogenesis remains to be elucidated. Nowadays, strong evidence shows a pivotal role for activated inflammatory cells in the initiation and perpetuation of the disease, and it is now generally accepted that APCs are key players in the pathogenesis of RA. The disease process of RA can be divided into several stages. During the early stages, synovial cell proliferation, infiltration of proinflammatory cells and defects in apoptosis lead to synovial thickening. As the disease process develops, the inflamed synovium invades the surrounding cartilage and bone, leading to complete joint destruction. Although the exact mechanism underlying RA pathogenesis is still unknown, the involvement of microorganisms has often been suggested. The fact that DNA viruses, including Epstein–Barr virus (EBV) [82], cytomegalovirus (CMV) [82] and parvovirus [83], have been shown to be present in RA synovium and fluid supports this hypothesis. Likewise, several reports suggest the possible involvement of bacterial products in the inflammatory circle of RA [84,85]. Although a direct link between the presence of pathogens and RA has not been demonstrated to date, the recent identification of TLRs sheds new light on the involvement of PAMPs in the articular inflammatory response.

Toll-like receptors in rheumatoid arthritis initiation & perpetuation

Interestingly, many animal models of arthritis applied TLR agonists for the induction of arthritis, long before TLRs had been identified. More recent studies have substantiated a role for TLRs in the initiation of experimental arthritis. For instance, TLR2 was found to play an essential role in the induction of streptococcal cell wall-induced arthritis, since both $TLR2^{-/-}$ and $MyD88^{-/-}$ mice demonstrated reduced signs of inflammation compared with control mice [86]. In addition, $TLR4^{-/-}$ mice have been shown to be less susceptible to collagen-induced arthritis [87], and ST2, an endogenous TLR4 inhibitor, attenuates disease severity in the same animal model [88]. Although informative, it should be realized that these animal models are artificial and do not provide an explanation for the role of TLRs in the pathogenesis of RA in the human setting.

To determine whether TLRs are involved in the initiation and/or perpetuation of RA in humans is extremely difficult. It is particularly complicated by the fact that the disease process is likely to have started long before the onset of symptoms, as demonstrated by the presence of citrullinated auto-antibodies and inflammatory markers. Nevertheless, a vast body of evidence links TLRs to the pathogenesis of RA. First, the expression of TLRs in synovial tissue from RA patients is increased compared with the expression found in osteoarthritis patients or healthy controls [89]. Second, DCs from RA patients are highly responsive to TLR ligands and produce higher levels of proinflammatory cytokines compared with control DCs [90]. Likewise, various groups have demonstrated that triggering of selective TLRs results in an increased production of chemokines in RA patients [91]. Taking into account that TLRs respond to endogenous danger signals, combined with the general belief that a variety of

those structures, such as HSPs, RNA from necrotic cells and hyaluronic acid, are present in the synovial compartment further supports the involvement of TLRs in RA. In addition, RA patients have high levels of circulating ICs and it was recently found that binding of IC to FcγRII can facilitate TLR7 and TLR9-mediated B-cell activation [92,93], a mechanism that is likely to function in DCs and macrophages as well. Interestingly, activated pDCs, which express both TLR7 and TLR9, are present in high numbers in synovial tissue of RA patients. However, pDCs isolated from synovial fluid display a more immature phenotype [94], which might be explained by the presence of ICs in synovial fluid that can inhibit DC maturation by binding to inhibitory FcR subtypes [95].

Is should be noted that activation of TLRs on other cell types besides APCs can significantly contribute to the inflammatory process, as, for instance, synovial fibroblasts produce a wide panel of chemokines, metalloproteinases and cytokines upon activation with TLR ligands [91,96,97]. It is tempting to speculate that the increased TLR expression and sensitivity for TLR ligands underlies both the pronounced state of activation of synovial tissue APCs and the high levels of pro-inflammatory mediators found in both synovial tissue and fluid. In addition, since pro-inflammatory cytokines can increase TLR expression, these circumstances might lead to an inflammatory response with a self-perpetual character and thereby contribute to the chronicity of RA (**Figure 3**).

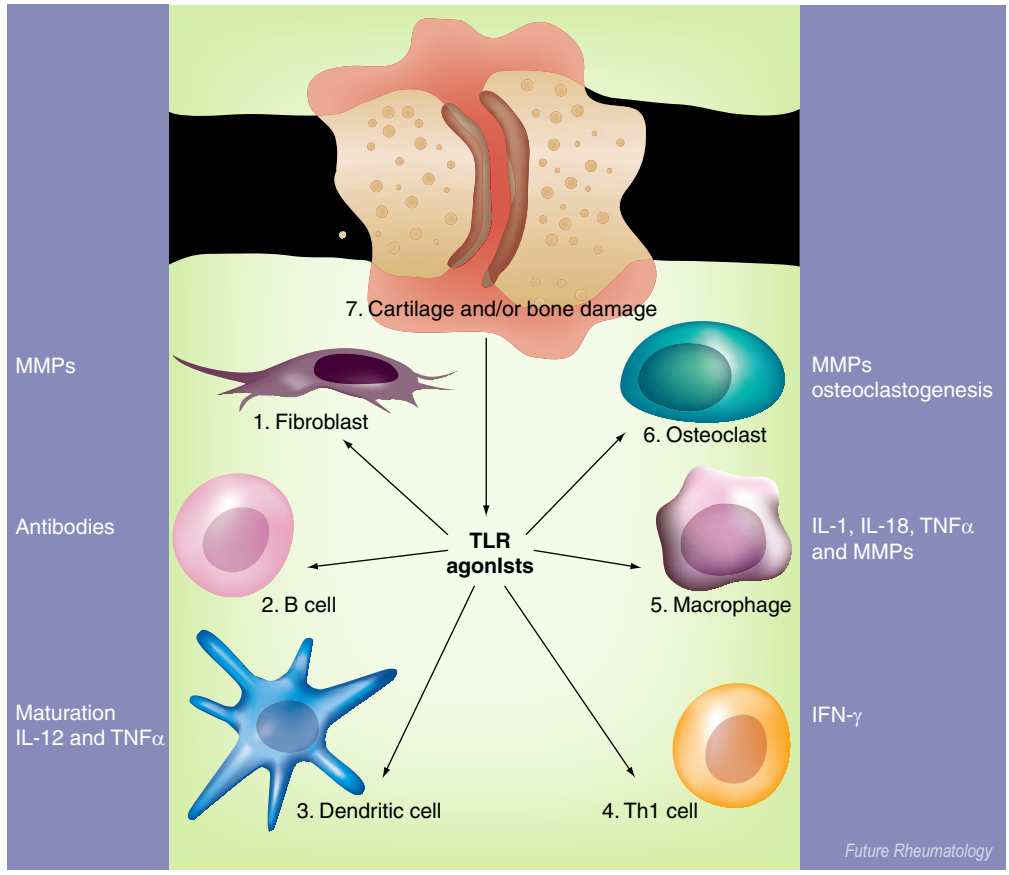
On another level, the use of association studies has provided some additional information on the role of TLRs in RA. The identification of the TLR4 Asp299Gly variant, which decreases responsiveness to LPS [98], enabled researchers to determine the influence of TLR4 in RA. Although earlier studies have failed to find an association between this TLR4 polymorphism and RA susceptibility [99,100], it was recently found to be present in a statistically significant lower frequency in RA patients [101]. However, there was no association with disease severity. These findings, although still requiring confirmation by research in independent cohorts, would suggest a role for TLR4 in the initiation, rather than perpetuation, of RA. The contribution of polymorphisms in other TLRs besides TLR4 has not been subjected to similar scientific scrutiny.

In conclusion, both animal models and human studies have yielded evidence for a role of TLRs in RA but have not identified the precise contribution of TLRs to RA susceptibility and/or chronicity. Future research is warranted to unravel the exact mechanism of involvement and may lead to the potential development of novel therapeutic interventions to battle this disabling chronic disease.

NOD-LRR proteins & arthritides

The recent discovery of the clear association between mutations in NOD2 and CD has unleashed research to investigate the potential role of NOD2 and other NOD-LRR proteins in susceptibility to a variety of arthritic conditions. To date, no association between mutations in NOD2 and susceptibility to RA [102,103], psoriatic arthritis [104,105] or ankylosing spondylitis [106] has been established, and the only clear association between NOD2 and arthritis is found in BS [71,72]. By contrast, the dramatic consequences of mutations in NALP3 in the previously mentioned autoinflammatory disorders MWS, FCAS and NOMID open up possibilities for currently unidentified role of members of this protein family in other inflammatory disorders that might seriously affect the joint. Recently, PBMC from patients with systemic onset juvenile idiopathic arthritis (SoJIA), a severe disease encompassing approximately 10% of all cases of arthritis that begin in childhood, were found to release large amounts of IL-1β upon activation [107]. IL-1RA administration to a small group of SoJIA patients resulted in complete remission in the majority of subjects, indicating the essential contribution of IL-1β to this disease. Remarkably, the efficacy of IL-1RA in these children is in sharp contrast to that of blocking TNF [107]. A significant body of evidence from the clinic as well as animal models has suggested IL-1β also plays a crucial role in the pathogenesis of RA [108,109]. However, despite the promising results of IL-1RA in SoJIA patients, the clinical effects of IL1-RA in adult RA are currently less impressive than these of TNF-α blockers [110–112]. To date, no studies have been published that have investigated the potential link between genetic variations in NALP family members and RA susceptibility. Although a number of NALPs have been identified as inflammasome components, it is not currently known whether other NALP family members also form these inflammatory caspase activating platforms. Furthermore, it is likely that many exogenous and endogenous NALP activators – and their potential role in immune disorders – will be

Figure 3. Model of Toll-like receptor agonists as catalysts in the rheumatoid arthritis inflammatory processes.



The mechanisms by which TLRs contribute the pathogenesis of rheumatoid arthritis (RA) are multifaceted. TLR activation of dendritic cell (DC) **(3)** is thought to be particularly important, since this leads to T-cell activation and often initiation of Th1 responses via release of IL-12. IFN-β release by Th1 cells can subsequently activate innate immunity, for instance macrophages **(5)**, that produce high amounts of IL-1, TNF-α, IL-18 and MMPs. TLR ligation can also directly activate T-cells **(4)** and B cells **(2)**, leading to increased cytokine production and the generation of high-affinity antibodies, respectively. TLR activation of fibroblasts **(1)** or osteoclasts **(6)** further results in release of pro-inflammatory cytokines, chemokines and/or MMPs. The mere presence of inflammatory mediators affects all the cell types present and further increases TLR expression. Collectively, the local inflammatory environment fuels the destructive processes into RA. Destruction in itself causes release of endogenous TLR agonist from damaged cartilage and bone **(7)**, leading to a self-perpetuating loop of inflammation, which contributes to the chronic character of RA. IFN: Interferon; IL: Interleukin; MMP: Matrix metalloprotease; Th: T helper; TLR: Toll-like receptor; TNF: Tumor necrosis factor.

discovered in years to come. It is tempting to speculate that future research in NALP biology, and perhaps identification of novel mutations in NALP encoding genes, will lead to new insights into the pathogenesis of RA and other arthritic conditions.

Conclusion

In recent years, TLRs and NOD-LRRs have unquestionably been identified as major regulators of innate and adaptive immunity. In addition, a vast body of data demonstrates their involvement in the initiation and aggravation of many immune disorders. Receptor polymorphisms, recognition of self-components and defective feedback mechanisms can all contribute to the pathogenesis of disease. A greater understanding of the exact activation and regulation of these receptors, and their signaling pathways could open up novel avenues for therapeutic strategies to combat autoimmune disorders.

Future perspective

To date, a large body of evidence suggests the involvement of TLRs in many inflammatory disorders; therefore, they are attractive targets for future therapies. Several strategies might be considered. One way of preventing TLR activation is blocking ligand–receptor interaction via application of specific monoclonal antibodies or TLR antagonists. These latter compounds can be ligand analogs that bind TLRs, but do not initiate signaling, or can interfere with ligand recognition by inhibiting the physical association between the ligand and accessory molecules [113,114]. However, blocking antibodies or antagonists might not inhibit binding of all recognized structures, owing to different ligand binding sites. In addition, binding sites of nearly all (endogenous) ligands are still obscure, which severely impairs the use of such antibodies in the clinic. Furthermore, the exact TLR and TLR-associated pathways that drive the inflammatory disorders remain to be elucidated. Since TLR activation does not consistently lead to induction of immunity, but can also contribute to tolerance, this is particularly important to determine. Until then, the use of antibodies or antagonists holds the danger of potentiating the inflammatory circle, rather than restoring tolerance.

Another way to prevent TLR activation would be to inhibit downstream signaling molecules, by using inhibitors of, for example, NF-κB or MAPKs [115–118]. These compounds can reduce proinflammatory cytokine production following TLR ligation, and have been shown to alleviate symptoms in several experimental animal models of arthritis. However, it should be realized that these compounds can severely impair the induction of immune responses that play a crucial role in the prevention of infections. An alternative option is to exploit one of the many endogenous inhibitory pathways, since a deranged function of these pathways could potentially underlie the chronic character of many autoimmune diseases. Further research characterizing the exact mechanisms of immune regulation, and the potential contribution of failing negative feedback to inflammatory disorders, could lead to novel therapeutic interventions aimed at restoring the delicate immune balance.

Finally, stimulation of specific TLRs can manipulate APC function, so that they gain tolerogenic capacities. Vaccination of autoimmune disease patients with these tolerogenic APCs could result in attenuation of symptoms and/or restoration of the immune homeostasis. Along the same line, repetitive triggering of DCs via TLRs has been shown to abrogate their proinflammatory capacity, supporting the idea that the effect of TLR signaling in directing APC behavior is heavily dependent on the timing, combination and quantity of TLRs triggered. The use of TLR-activated, immune-stimulatory DCs has already shown promising results in the battle against malignancies [119,120]. In the case of inflammatory disorders, stimulation with immunosuppressive compounds or cytokines (e.g., vitamin D3, dexamethasone or IL-10) in combination with TLR stimulation might be more effective. Treatment with *ex vivo* instructed, tolerogenic DCs requires a stable DC phenotype that is not affected by the proinflammatory environment these cells will encounter *in vivo*.

Future research uncovering the exact mechanisms of TLR and NOD-LRR activation, and their feedback mechanisms, might be extremely rewarding. Once a thorough understanding is achieved, development of immune intervention strategies targeting these molecules or their signaling pathways could become powerful approaches in the battle against immune disorders.

Acknowledgements

This work was supported by NWO grants 901.10.092 and 918.66.615. The authors would like to thank Dirk J de Jong for helpful discussions.

Executive summary

Toll-like receptor function & signaling

- Toll-like receptors (TLRs) are pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs) and endogenous danger signals.
- TLR ligation results in activation of diversified signaling pathways and a tailored immune response.
- Endogenous feedback mechanisms attenuate TLR signaling to prevent excessive inflammatory responses.

TLR expression & localization

- TLRs are expressed in various cells and tissues that form the interface between internal and external milieu.
- TLR localization within cells and tissues is aimed at instant recognition of pathogens, while minimizing the chance of response to self-components.

Executive summary

Role of TLRs in adaptive immune responses

- Activation of different TLRs on dendritic cells regulates T-cell differentiation.
- Some pathogens have evolved to avoid TLR activation or exploit TLRs to induce immune deviation.

NOD-LRR protein family

- Nucleotide-binding oligomerization domain (NOD) leucine-rich repeat (LRR) proteins are intracellular proteins with a LRR domain similar to TLRs.
- NODs recognize different peptidoglycan moieties and synergize with TLRs for the production of cytokines.
- Specific NOD-LRR subfamily members are components of the inflammasome, which regulates interleukin (IL)-1β processing.

TLRs & NOD-LRRs in immune disorders

- Cooperation between TLR9 and FcγRIIa results in inflammatory responses against systemic lupus erythematosus (SLE) DNA-imunno complexes.
- TLRs are important contributors to the intestinal inflammation in various mouse models.
- TLR and NOD2 polymorphisms are associated with increased susceptibility to Crohn's disease.
- Mutations in NALP3 lead to a variety of autoinflammatory disorders.

Involvement of TLRs & NOD-LRRs in arthritides

- TLRs contribute to joint inflammation in various animal models of arthritis.
- In humans, expression of TLRs in rheumatoid arthritis (RA) synovial tissue is increased.
- In RA, TLR activation on several cell types results in production of higher levels of cytokines and chemokines.
- The presence of inflammatory mediators and endogenous ligands in inflamed joints may lead to a self-perpetuating inflammatory loop, contributing to the chronic character of RA.

Future perspective

- Future research aimed at unraveling TLR and NOD-LRR activation and feedback pathways could contribute to the development of novel therapeutics to alleviate various immune disorders.

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