cells. A high concentration of IL-10 in the tumor microenvironment may stimulate NK cells to lyse cancer cells, leading to increased availability of tumor-associated antigens and delivery of biologically active molecules, such as heat-shock proteins, needed for the activation of DCs and for effective priming of CTLs against tumor-associated antigens.[33]

We need good manufacturing practices to purify adjuvants such as BCG-CWS for translational research and to coadministrate with more personalized peptide vaccines as a future challenge. In conclusion, our results suggest that BCG-CWS immunotherapy following radical surgery for NSCLC improves overall survival without compromising quality of life.

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Toll-like Receptors

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Advanced article

Article Contents

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Toll-like receptors (TLRs) are mainly expressed on antigen-presenting cells (APCs) and function as adjuvant receptors that modulate cellular immunity. Their pleiotropic functions depend on a variety of signals. Ten members of the TLR family in humans and 10 ~ 20 TLRs in other vertebrates cope with microbial pattern molecules, that is, TLR agonists. In addition, a number of molecules negatively or positively regulate the TLR signal pathways. TLR agonists are capable of activating either of the two main TLR pathways driven by the adaptors, myeloid differentiation primary-response gene 88 (MyD88) and TRIF (TIR-containing adaptor-inducing interferon-β TIR-containing adaptor molecule-1, TICAM-1), which confer differential maturation modes on myeloid (mDCs) and plasmacytoid dendritic cells (pDCs). Natural killer (NK), CD8 T, CD4 T and B cells are activated through matured dendritic cells. TLRs are therefore involved in not only inducing the acute phase of inflammatory responses but also raising the acquired immune responses including allergy, cancer and infectious immunity.

Introduction

The Toll-like receptor (TLR) family is a fundamental receptor family involved in sensing pathogen-associated molecular patterns (PAMPs) to raise alarm signals in host cells. This family consists of multiple members that have different primary structures and are distributed in a wide variety of vertebrates and invertebrates. These receptors are localized in host cells and signal the presence of molecular patterns specific to microbial cells and denatured material of host cellular origin. Current understanding of the innate immune system is that TLRs, in concert with other innate immune systems, protect the host from life-threatening infections. Rapid responses are essential for host survival during the early phase of infection. Many intracellular mediators and antimicrobial factors are induced by TLR signalling in various species. **See also**: Pattern Recognition Receptor; Vertebrate Immune System: Evolution

Prompt immune responses for overcoming acute infections usually involve TLR signalling in innate immune cells before an increase in the adaptive immunity. In particular, vertebrates have an innate system with professional antigen-presenting cells (APCs), including dendritic cells, which defend the host against pathogen attack by coordinating with the adaptive immune system. Adaptive immunity is mediated by B and T lymphocytes. These lymphocytes carry rearranged receptors that can discriminate various antigens with high affinity. Antigen-specific lymphocytes survive in the host for a long time and remain on 'stand-by' for future rechallenge by antigens. Thus, adaptive immunity is characterized by specificity and memory.

However, accumulating evidence pointed that cell-mediated immunity (including T, B and natural killer (NK) cells) is augmented by the maturation of dendritic cells and that TLRs are critical to the process of dendritic cell maturation. Antibody production by B cells and the levels of many effectors, such as CD8 + cytotoxic T lymphocyte (CTL), NK, CD4 + (T-helper cell 1 (T_H1), T_H2, T_H17 and regulatory T cell (T_{reg}), are enhanced or modulated by matured dendritic cells in response to TLR agonists. There are a number of subsets of dendritic cells such as myeloid (mDCs) and plasmacytoid dendritic cells (pDCs). The mechanisms by which dendritic cells mature through TLR stimulation have been elucidated in the last few years. **See also**: Dendritic Cells (T-lymphocyte Stimulating); Follicular Dendritic Cells (B Lymphocyte Stimulating)

TLRs in innate immune cells can discriminate a variety of microorganism-derived molecular structures and are

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therefore also called pattern-recognition receptors. Upon recognition, mammalian TLRs can activate APCs to produce pro-inflammatory cytokines and augment costimulatory molecule expression and can also express case-sensitive molecules, thereby driving specific effectors and eventually shaping cell-mediated immunity. Here, we will review the functions and signalling mechanisms of TLRs. **See also**: Antigen-presenting Cells

Characteristics of the TLR System

TLRs are named after the Drosophila protein 'Toll'. TLRs and Tolls have similar structural features and functional properties. Both TLRs and Tolls are type-I transmembrane proteins consisting of an *N*-terminal extracellular domain rich in leucine residues, namely, leucine-rich repeats (LRRs) and a *C*-terminal cytoplasmic domain designated as the Toll/interleukin 1 receptor homologous (TIR) domain. TIR domains are essential for coupling to TIR-containing adaptor molecules in order to trigger signal transduction. In both vertebrates and invertebrates, molecules involved in the signalling cascades are also highly preserved among TLRs (**Figure 1**) and within the Toll system.

Toll signalling can lead to the expression of an antifungal peptide, drosomycin, and Toll-defective mutant flies succumb to fungal infection. Janeway's group first characterized human TLR, now called TLR4, and found that it could induce cytokine production and upregulate costimulatory molecules for mDC maturation (Medzhitov *et al.*, 1997). Thereafter, other mammalian TLRs have been found to activate a variety of immune responses. Thus, both Toll and TLRs have common features in host defence against pathogens.

However, the mechanism by which TLRs recognize pathogen-associated patterns is distinct from that employed by Tolls. In flies, pathogens are recognized in the haemolymph, and protease cascades are activated to cleave a host-derived soluble protein Spaetzle. Toll recognizes the cleaved form of Spaetzle. In contrast, TLRs can directly recognize a variety of microbe-derived pattern molecules. Thus, mammals sense pathogens on the cell membrane, whereas flies discriminate them in the circulation. The complement system consists of serine protease cascades in the plasma, which in turn activate effector molecules. In invertebrates, the Toll and complement systems are conceptually similar, whereas in vertebrates, a unique TLR system has been developed for host defence, resulting in two distinct innate systems, the TLR and complement systems.

TLRs and Their Ligands

Ten TLR family members have been identified in humans (**Table 1**). At least one ligand has been identified for each TLR, with the exception of human TLR10. These ligands

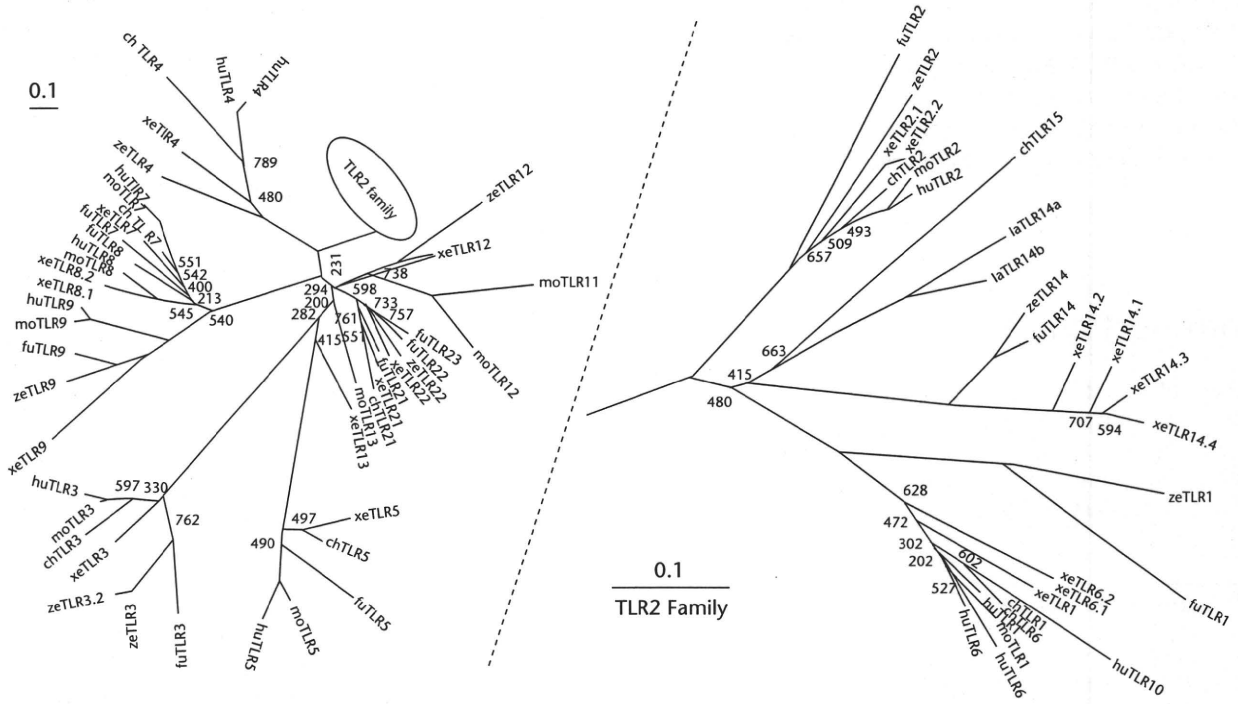


Figure 1 Evolutional view of TLRs. Unrooted phylogenetic tree of TLRs in vertebrates. The tree was constructed by the Clustal W programme. The relationships were calculated on the basis of the amino acid sequences of TIR domains. Bootstrap values (<800) were indicated. Asterisk indicates trichotomy. ch, chicken; fu, fugu; hu, human; la, lamprey; mo, mouse; xe; *Xenopus tropicalis*, ze; zebrafish.

Table 1 Properties of human Toll-like receptors

HuTLR	Amino acids	Molecular weight	Adaptors	Ligands	Modes	Chromosomes
TLR1	786	85 kDa	M-1/M-2	triacyl BLP	M-type	4p14
TLR2	784	82 kDa	M-1/M-2	PGN, BLP	M-type	4q32
TLR3	904	110 kDa	T-1 (M-1)	dsRNA	T/M-type	4q35
TLR4	839	95 kDa	M-1/M-2	LPS, Taxol	M/T-type	9q32
TLR5	858	95 kDa	M-1	flagellin	M-type	1q41
TLR6	796	89 kDa	M-1/M-2	diacyl BLP	M-type	4p14
TLR7	1049	118 kDa	M-1	ssRNA	M/T-type	Xp22
TLR8	1059	112 kDa	M-1	ssRNA	M/T-type	Xp22
TLR9	1032	120 kDa	M-1	CpG DNA	M/T-type	3p21

Note: TLR1 and TLR6 are members of the TLR2 subfamily and, in conjunction with TLR2, recognize more precise BLP patterns to activate the TLR2 pathway. Mice express no counterpart of huTLR8.
M-1, MyD88; M-2, Mal/TIRAP; T-1, TICAM-1; T-2, TICAM-2; M-type, MyD88-dependent NF-κB activation pathway; T-type, IRF-3/IRF-7-mediated type I IFN-inducing pathway.

are absent in the host, but they are present in certain groups of microorganisms. Microorganisms carry TLR ligands, irrespective of their pathogenicity. Notably, recent pattern-searching studies on TLRs have identified endogenous ligands for TLRs. These intrinsic ligands are released secondary to host cell damage or noninfectious inflammation (Kono and Rock, 2008). Since all TLR ligands have potent immunostimulatory activity, TLRs can also be regarded as adjuvant receptors. They can be divided into subfamilies based on their amino acid structures and characteristics of their ligands (**Figure 2**). Structurally related TLRs tend to discriminate similar types of ligands. First, lipid ligands are recognized by TLR4, TLR2, TLR1 and TLR6. Second, flagellin, a protein ligand, is recognized by TLR5, which is distantly related to other TLRs. Finally, nucleic acids are discriminated by TLR3, TLR7, TLR8 and TLR9.

Mouse and teleost fish have unique TLRs in addition to orthologs of human TLRs. TLR11, TLR12 and TLR13 in mice and TLR21 and TLR22 in fish are members of the TLR20 family: these resemble each other in terms of structure but have been lost in humans. TLR11 and TLR21 recognize Gram-negative bacterial patterns (Yarovinsky *et al.*, 2005), while TLR22 senses the double-stranded ribonucleic acid (dsRNA) of viruses (Matsuo *et al.*, 2008).

Recent findings on retinoic acid–inducible gene (RIG)-I-like receptors (RLRs) and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) indicate that a part of the cellular response induced by TLR ligands can be attributed to intracytoplasmic pattern-recognition receptors. **See also**: Innate Immune Mechanisms: Nonself Recognition; Pattern Recognition Receptor

TLR4

The first TLR ligand to be identified was lipopolysaccharides (LPS). LPS are polysaccharides with acyl chains and constitute the outer leaflet of the outer membrane of Gram-negative bacteria (Raetz and Whitfield, 2002). Positional cloning of a mutation in the LPS-hyporesponsive mouse strains C3H/HeJ and C57BL/10ScCr led to the identification of TLR4 as an LPS-sensing molecule (Poltorak *et al.*, 1998; Qureshi *et al.*, 1999). These findings were corroborated in TLR4-deficient mice (Hoshino *et al.*, 1999), which do not respond to LPS.

However, TLR4 alone is not sufficient for LPS sensing and signalling. LPS is trapped by the LPS-binding protein (LBP) in the serum and transported to target cells. Target cells express another LPS-binding protein CD14. CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein that retains LPS on the cell surface. Although these molecules are not essential for the LPS response, they have an important role in amplifying LPS responses. LPS responses are profoundly impaired in LBP- or CD14-deficient mice (Haziot *et al.*, 1996; Jack *et al.*, 1997). MD-2 is physically associated with the extracellular domain of TLR4 and is essential for TLR4 recognition of its ligand. MD-2-deficient mice do not respond to LPS and survive endotoxin shock (Nagai *et al.*, 2002). The structure of MD-2 has revealed the presence of a hydrophobic pocket that accommodates acyl chains of LPS (Ohto *et al.*, 2007; Kim *et al.*, 2007). The LPS/MD-2 complex is thought to induce TLR4 dimerization and activate LPS signalling. The LPS sensor is a heterodimer consisting of TLR4 and MD-2.

Mouse TLR4, not human TLR4, can also recognize taxol (Ding *et al*, 1990), which is an antitumor agent derived from a plant. Taxol is a diterpene that is structurally unrelated to LPS. Recognition of taxol also requires MD-2 (Kawasaki *et al.*, 2000). Although mutation analysis of MD-2 residues revealed that the same residues are necessary for the responses to LPS and taxol, the requirement of some residues in MD-2 differ, indicating that LPS and taxol are recognized through distinct domains.

TLR4 recognizes membrane proteins of virus origin, the F protein of respiratory syncytial virus (RSV) (Kurt-Jones *et al.*, 2000). Several host-derived molecules, including heat shock proteins (HSPs) and oligosaccharides such as hyaluronic acids, are reported to be TLR4 ligands. HSPs are secreted from injured cells and hyaluronic acids are degraded during inflammatory responses. This suggests the interesting possibility that TLR4 also functions to

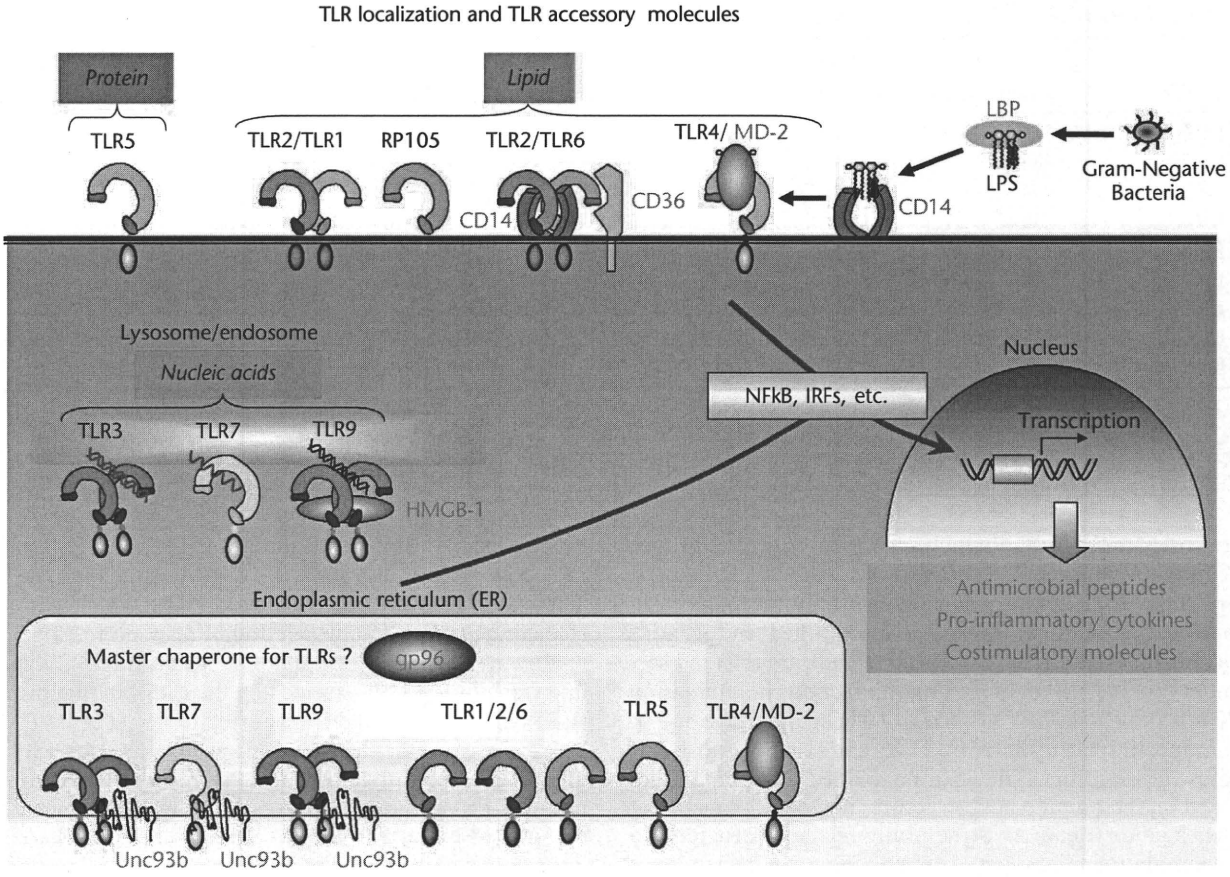


Figure 2 TLR family members and their ligands. TLRs can recognize a variety of microorganism-derived components. Lipid ligands, derived mainly from membrane components, are recognized by TLR2 and TLR4. TLR2 recognizes its ligands by forming heterodimers with TLR1, TLR6 or possibly TLR10. TLR4 requires another secreted molecule, MD-2 for ligand recognition. CD14 is required for initial recognition of LPS/LBP complex. A flagella-derived protein, flagellin, is recognized by TLR5. These TLRs are localized on the cell surface as mature forms. Nucleic acids and their relatives are recognized by TLR3, TLR7 or TLR9, which are localized in cytoplasmic compartments. TLR proteins are present as immature forms in ER and sorted to their working places with putative shaperons. TLR3, TLR7 or TLR9 are complexed with Unc93b in ER.

stimulate innate immunity under certain alert conditions. However, it should be noted that some HSP preparations are contaminated with LPS and such results should therefore be interpreted with caution.

TLR2 and its relatives, TLR1 and TLR6

A number of bacterial lipoproteins function as TLR2 ligands. Ligands include peptidoglycans (PGNs) from Gram-positive bacteria, bacterial and mycoplasmal lipopeptides, GPI-anchors from *Trypanosoma cruzi*, zymosan from yeast, and lipoarabinomannan from mycobacteria. TLR2 can also recognize LPS from *Leptospira* or *Porphyromonas gingivalis*. The LPS from these organisms are atypical and differ structurally from the LPS of Gram-negative bacteria. Thus, TLR2 is involved in the recognition of different bacterial membrane lipid components and PGNs with muramyl dipeptide (MDP).

The hyperlipidaemia-induced increase in TLR2 expression in vascular endothelial cells leads to the recognition of unknown host ligands in atherosclerosis lesions in mouse/human vessels and exacerbates atherosclerosis in LDL −/− mice (Mullick *et al.*, 2008). Amyloid beta in the neurons of patients with Alzheimer disease may also act as an endogenous TLR2 ligand (Jana *et al.*, 2008).

The broad recognition spectrum of TLR2 can be ascribed, at least partly, to the heterodimerization of TLR2 with TLR1 or TLR6, first suggested as Ozinsky *et al.* (2000). TLR6-deficient mice did not respond to mycoplasma but were sensitive to bacterial lipopeptides, while TLR2-deficient mice did not react to either. Both types of lipopeptides carry an *N*-terminal cysteine residue, which is either diacylated (in mycoplasmal lipopeptides) or triacylated (bacterial lipopeptides), and this difference in acylation is recognized by TLR6 and TLR1, respectively. Taken together, heterodimer formation is important for broader microbial recognition by TLR2.

The MDP of Gram-positive bacteria is a ligand for NOD2 and diaminopimeric acid (DAP)-containing PGN from Gram-negative bacteria is a ligand for NOD1 (Benko *et al.*, 2008; Franchi *et al.*, 2009). The TLR2 and TLR4 responses are amplified in some cases in concert with

intracellular NOD signalling. **See also**: Innate Immune Mechanisms: Nonself Recognition

Birds and fish possess the putative TLR2 members TLR14 and TLR15, but their ligands and properties remain unknown.

TLR5

Flagellin is a protein component of the flagella and is a propulsive structure used by some bacteria to move through a liquid medium. Flagellin can stimulate intestinal epithelial cells and induce interleukin 8 (IL-8) production and nitric oxide synthesis. Enforced expression of TLR5 can confer Chinese hamster ovary cells with the ability to respond to flagellin. Furthermore, mutant bacteria lacking flagellin fail to activate TLR5 signalling. Thus, TLR5 is involved in the recognition of bacterial flagellin (Hayashi *et al.*, 2001). A stop codon polymorphism in TLR5 is associated with susceptibility to pneumonia caused by *Legionella pneumophila*, which is flagellated. Part of the flagellin recognition response is mediated by IPAF of the NLR family in the cytoplasm.

TLR5 is expressed not on the apical but on the basolateral surface of epithelial cells (Gewirtz *et al.*, 2001). This expression pattern enables the host to respond only to pathogenic bacteria that can translocate across the epithelia. Thus, the TLR5 expression pattern is critical for the host to exert effective mucosal immunity.

Teleost fish possess a soluble form of TLR5, named TLR5S, which lacks the TIR domain, to amplify the membrane TLR5-mediated flagellin response, leading to endotoxin-like shock in fish (Tsujita *et al.*, 2004).

TLR3

RNA virus infection leads to the generation of dsRNAs, which can stimulate immune responses. Their synthetic mimic polyinosinic–polycytidylic acid (poly(I:C)) also exhibits similar adjuvant activity. Enforced expression of TLR3 confers the ability to respond to dsRNAs (Alexopoulou *et al.*, 2001). This induces interferon-regulatory factor 3 (IRF-3) activation, leading to interferon β IFNβ production. TLR3.7, an antihuman TLR3 monoclonal antibody, blocks IFNβ production by human fibroblasts (Matsumoto *et al.*, 2003). This monoclonal antibody (mAb) can be used as a probe to determine the subcellular localization of TLR3 as early endosomes in dendritic cells and on the cell surface of fibroblasts/epithelial cells (Matsumoto *et al.*, 2003). Fibroblasts bind the dsRNA of viruses on their cell surface to activate IRF-3 and produce IFNβ. However, exogenously added dsRNA fails to induce type I IFNs in mDCs. Although TLR3 has an IFN-inducing role in the local epithelial milieu, TLR3 may not be the main IFNβ inducer in the systemic response to dsRNA. In this context, TLR3-deficient mice still respond to dsRNAs. A recent finding shows that the RLRs of RIG-I and melanoma differentiation-associated gene 5 (MDA5) are localized to the cytoplasm and respond robustly to dsRNA. Current understanding of the dsRNA-sensing mechanism is that dsRNA generated in the cytoplasm preferentially activates the RLR-mediated IFNβ-inducing pathway, while dsRNA liberated from infected cells is recognized by TLR3 in either bystander epithelial or myeloid cells. In mDCs, dsRNA incorporated in phagosomes reaches endosomal TLR3 to activate the extrinsic IFNβ-inducing pathway (Ebihara *et al.*, 2008). The latter might occur when cell debris with viral dsRNA is liberated from virus-infected cells and internalized into mDCs to encounter endosomal TLR3 (Ebihara *et al.*, 2008). The intrinsic and extrinsic IFN-inducing pathways converge upon the IRF-3-activating kinases (Sasai *et al.*, 2006). **See also**: Innate Immune Mechanisms: Nonself Recognition; Interferons; Pattern Recognition Receptor

TLR3 expression is limited in myeloid and fibroblasts/epithelial cells. TLR3 with ligand stimulation can drive lymphocyte activation; that is, NK cells and CD8+ CTLs are reciprocally activated through poly(I:C)–mediated mDC maturation. CD4+ T cells are also activated in response to poly(I:C), suggesting that TLR3 is mainly involved in dsRNA-induced cellular immune responses.

TLR7 and TLR8

TLR7 and TLR8 are located on the X-chromosome and are highly homologous to each other (**Figure 2**). Imidazoquinoline derivatives, such as imiquimod and resiquimod (R848), were first identified as TLR7 ligands (Hemmi *et al.*, 2002). Both substances are synthetic low-molecular-weight compounds that can induce the production of a variety of cytokines, including type I IFN. TLR7 is also required for the effects of other synthetic anticancer chemicals such as loxoribine (7-allyl-8-oxoguanosine) and bropirimine (2-amin-5-bromo-6-phenyl4(3)-pyrimidinone). Furthermore, certain guanosine analogues can also stimulate TLR7 signalling. The molecular structure of these TLR7 ligands appears to be similar to that of nucleic acids. Indeed, TLR7 was found to be essential for the recognition of single-stranded ribonucleic acids (ssRNAs) such as *influenza virus*, *respiratory syncytial virus*, *Sendai virus*, and *vesicular stomatitis virus* (Heil *et al.*, 2004; Diebold *et al.*, 2004) by dendritic cells and macrophages. TLR8 has been shown to respond to ssRNA in humans but its response in mice has not yet been clarified. In humans, TLR7 is expressed in pDCs, whereas TLR8 is expressed in mDCs. TLR7 activation in pDCs induces the production of a large amount of type I IFN (Ito *et al.*, 2002). TLR7 also responds to self-derived nucleic acids, and in mice models (Christensen *et al.*, 2006), it is shown to predispose individuals to autoimmune diseases.

TLR9

In addition to lipid or protein components, bacterial deoxyribonucleic acids (DNAs) can also efficiently stimulate immune responses. It was originally reported by Tokunaga *et al.* that DNA elements are responsible for the

adjuvant activity of bacille Calmette–Guérin (BCG) extracts. An unmethylated CpG dinucleotide motif occurs more frequently in bacterial DNAs than in mammalian DNAs. Furthermore, oligodeoxynucleotides containing an unmethylated CpG motif (CpG DNA) may exhibit strong immunostimulatory activity. The adjuvant activity of CpG DNA is abolished in TLR9-deficient mice, indicating that TLR9 is a critical factor in the recognition of CpG DNA (Hemmi *et al.*, 2000). DNA viruses such as the *herpes simplex* and *cytomegalovirus* viruses contain an unmethylated CpG motif and can activate immune cells through TLR9 (Zhang *et al.*, 2007). Thus, TLR9 is critically involved in antiviral immunity. CpG DNA is incorporated into early endosomes and transported to a tubular lysosomal compartment, where it activates TLR9 signalling through direct interaction with TLR9 (Latz *et al.*, 2004). TLR9 resides in the endoplasmic reticulum (ER) at the steady state and translocates to an endolysosome upon ligand stimulation. Unc93B1, an ER protein, plays a critical role in TLR9 trafficking (Kim *et al.*, 2008). TLR9 is proteolytically cleaved in endolysosomes, and this cleavage is required for TLR9 signalling (Matsumoto *et al.*, 2008; Ewald *et al.*, 2008; Park *et al.*, 2008).

TLR11, TLR21 and 22

Mice express TLR11, TLR12 and TLR13 in addition to analogues of human TLR1 ~ TLR7 and TLR9. Cells expressing TLR11 fail to respond to known TLR ligands but respond specifically to uropathogenic bacteria (Zhang *et al.*, 2004). Mice lacking TLR11 are highly susceptible to kidney infection caused by uropathogenic bacteria. The first identified TLR11 ligand has been recently described as a profilin-like protein from *Toxoplasma gondii* (Yarovinsky *et al.*, 2005). TLR11 activates the myeloid differentiation primary-response gene 88 (MyD88) pathway and induces IL-12, which may explain why a *Toxoplasma gondii* infection induces high levels of IFNγ. The ligands of TLR12 and TLR13 have not yet been identified.

Fish express TLR21 and TLR22 together with all other human TLR analogues of human with the exception of TLR4. TLR21 recognizes the PGN of Gram-negative bacteria, which contains DAP. TLR21 is localized on the cell surface and recognizes a DAP-containing moiety of PGN independent of TLR2, a representative PGN receptor. TLR21 is linked to the adaptors MyD88 and TRIF (TIR-containing adaptor-inducing interferon-β; TIR-containing adaptor molecule-1, TICAM-1) to induce the activation of nuclear factor-κB (NF-κB) and IFNβ promoter, similar to the functions of human TLR4. Thus, fish can discriminate between infection by Gram-negative and Gram-positive bacteria on the basis of structural differences in the PGN peptide stretch. TLR22 recognizes long dsRNAs present on the cell surface and activates the TRIF (TICAM-1) pathway (Matsuo *et al.*, 2008). Unlike TLR3, it is exclusively localized on the cell surface, suggesting that humans have lost TLR22 but have replaced it with the surface-expressed form of TLR3. TLR22 is widely distributed in fish and facilitates the adaptation of the organism to the water environment that contains dsRNA viruses. Fish possess TLR20 family members other than TLR21 and TLR22, but the ligands and functional properties of these are unknown.

Biological Function of TLRs

TLRs engage in acute inflammatory signalling in cells by inducing pro-inflammatory cytokines such as IL-1, IL-6 or tumour necrosis factor α (TNFα). These cytokines then activate monocytes or neutrophils, establishing inflammatory responses to eradicate pathogens. mDCs mature through their TLRs to reciprocally activate NK cells, which function as effector cells in the milieu of inflammatory lesions. Furthermore, antigen-dependent lymphocyte proliferation is critically modulated through the maturation of mDCs. An outline of TLR signalling–derived output in mDCs is shown in **Figure 3**.

Link between innate and adaptive immunities

TLR signalling also activates APCs, especially mDCs, to establish protective adaptive immunity (Iwasaki and Medzhitov, 2004). T cells cannot be activated merely by T-cell receptor (TCR) signalling and require additional signals from mDCs for clonal expansion. TLR signalling in mDCs provides this second signal by upregulating the expression of both class I and II major histocompatability complex (MHC) molecules, costimulatory molecules such as CD80 or CD86, NK-activating ligands including Rae-1 and unknown molecules supporting the link between mDCs and other effector cells. Effector cells, with the exception of NK cells, harbour rearranged receptors for peptide-mounted MHCs, and thereby belong to the acquired immune system. CD8+ T cells, CTL, CD4+ T cells, T_{reg}, T_H1, T_H2 and T_H17 lymphocytes are differentially induced by mDC TLR signals. Furthermore, TLR-stimulated mDCs produce IL-12 or IL-18, which further skew T_H cells to differentiate into T_H1 and T_H2 (see Glossary, T_H1 and T_H2) cells. TLR-induced IL-6 regulates the induction of T_{reg} cells; hence T_{reg} development is inhibited by TLR-stimulated mDCs or augmented by TLR2-stimulated mDCs (Pasare and Medzhitov, 2003). TLR2/TLR4 helps mDCs induce IL-23, which facilitates the activation of T_H17 cells. Thus, both CD4 and CD8 T-cell differentiation is finely regulated by TLR-stimulated dendritic cells (Pasare and Medzhitov, 2004). For B-cell activation and antibody production, generation of T-dependent antigen-specific antibody responses requires activation of TLRs in B cells in addition to CD4+ T-cell help. However, TLR signalling may not fully explain the action of adjuvant activating the TLR pathways (Gavin *et al.*, 2006).

Antiviral immunity

Type I IFNs are critical immunoregulatory cytokines that induce mDC maturation and augment immune responses

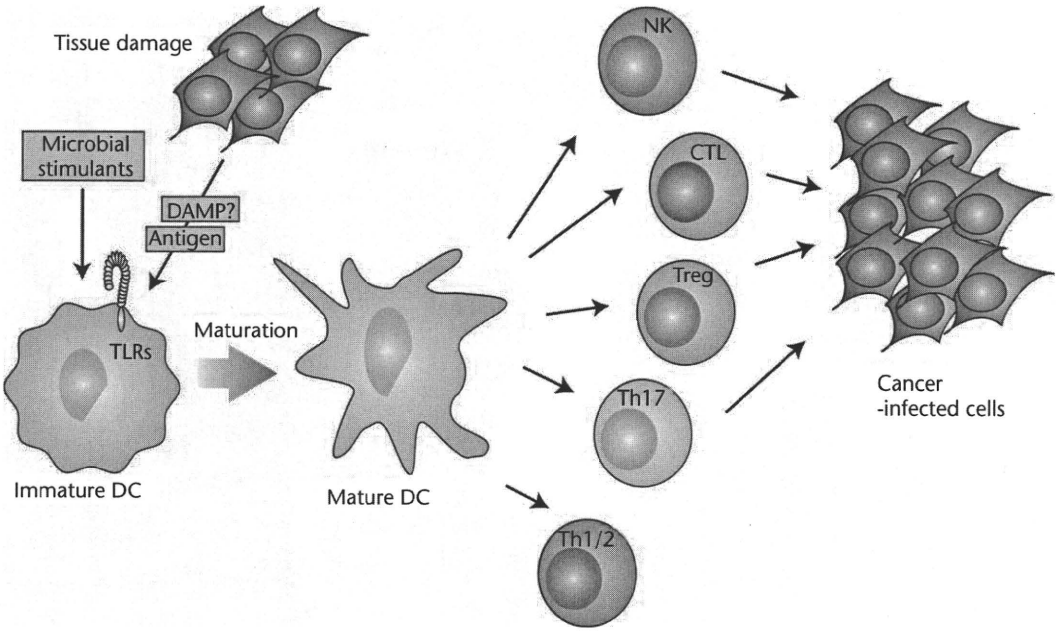


Figure 3 Overview of dendritic cell TLR output. Myeloid dendritic cells (mDCs) mature in response to TLR signalling. The MyD88 and TRIF (TICAM-1) pathways in mDCs are critical for driving cell-mediated immunity. They contribute mDC maturation to establishing antigen-specific T-cell proliferation, CTL and T_H1 responses. mDC-mediated NK-cell activation is characterized by its TRIF-dependent properties which activate the transcription factor IRF-3. Induction of variable CD4 T cells such as T_H2, T_H17 and T_{reg} are partly attributable to mDC maturation through the TLR pathways. NK and T_{reg} are also modulated by the cytoplasmic IPS-1 pathway that activates the IPS-1 pathway for IRF-3 activation and IL-6 production.

against viral infection. Type I IFNs include more than 10 IFNα and a single IFNβ. They act through the same receptor, i.e., IFNα/βR, but expression of their genes requires distinct sets of transcription factors. More importantly, IFN-inducing TLRs can be subdivided according to their adaptor usage. TLR3 and TLR4 recruit TRIF (TICAM-1) to induce IFNβ production in response to their viral ligands in mDCs (Oshiumi *et al.*, 2003b; Yamamoto *et al.*, 2003a). TLR7 and TLR9, which recognize nucleic acids or nucleic acid–like structures in the endosome, recruit MyD88, leading to IFNα production (Hemmi *et al.*, 2000, 2002).

pDCs are a unique subset of dendritic cells that produce IFNα. In humans, pDCs selectively express TLR7 and TLR9 and secrete IFNα in response to their ligands. In mice, although mDCs and pDCs express TLR9, only pDCs produce IFNα through TLR9 signalling involving MyD88 and IRF-7 (Honda *et al.*, 2005,). Both human and mouse mDCs are responsible for type I IFN induction via TLR3 and RLRs in response to poly(I:C). Thus, dendritic cell subsets exhibit differential modes of type I IFN induction that depend on TLR adaptor selection in cells.

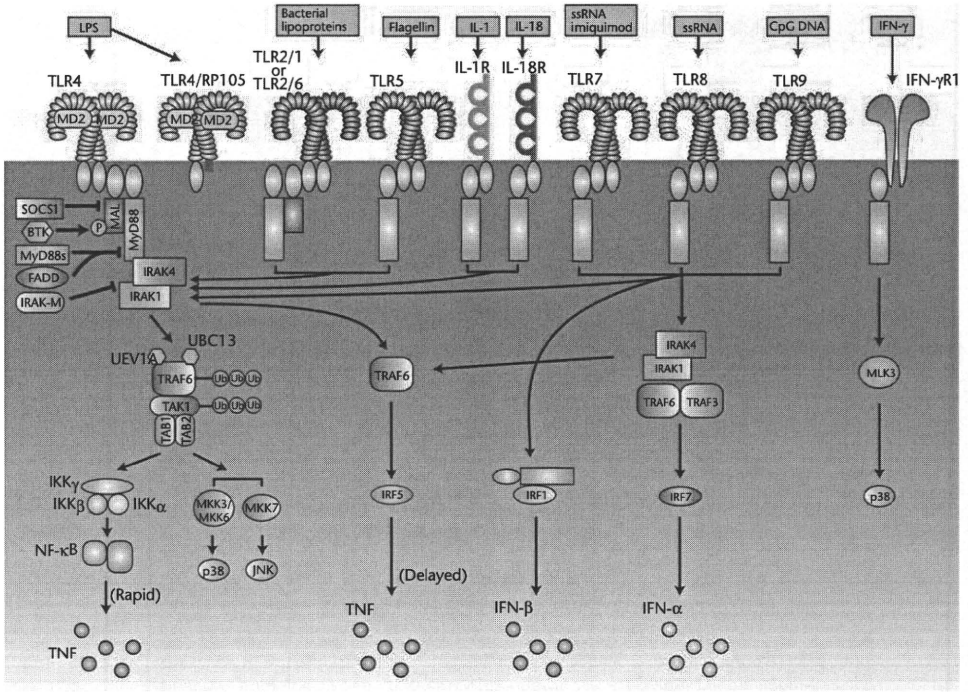
Signalling Through TLRs

Four adaptors that have the TIR domain can couple with TLRs. MyD88 and TRIF (TICAM-1) are functional adaptors that forward signals to downstream molecules. Two of these, TIR domain–containing adaptor protein/MyD88 adaptor–like (TIRAP/Mal) and TRIF-related adaptor molecule (TRAM) (TICAM-2), are bridging adaptors that link TLRs to MyD88 and TRIF, respectively. The adaptor-selecting repertoires of each TLR are shown in **Figure 4a** and **b**. All human TLRs, with the exception of TLR3, recruit MyD88. TLR2 and TLR4 indirectly bind MyD88 through TIRAP/Mal) (Horng *et al.*, 2002; Yamamoto *et al.*, 2002). TLR7 and TLR9 directly bind MyD88. TLR3 and TLR4 recruit TRIF either directly or indirectly through TRAM.

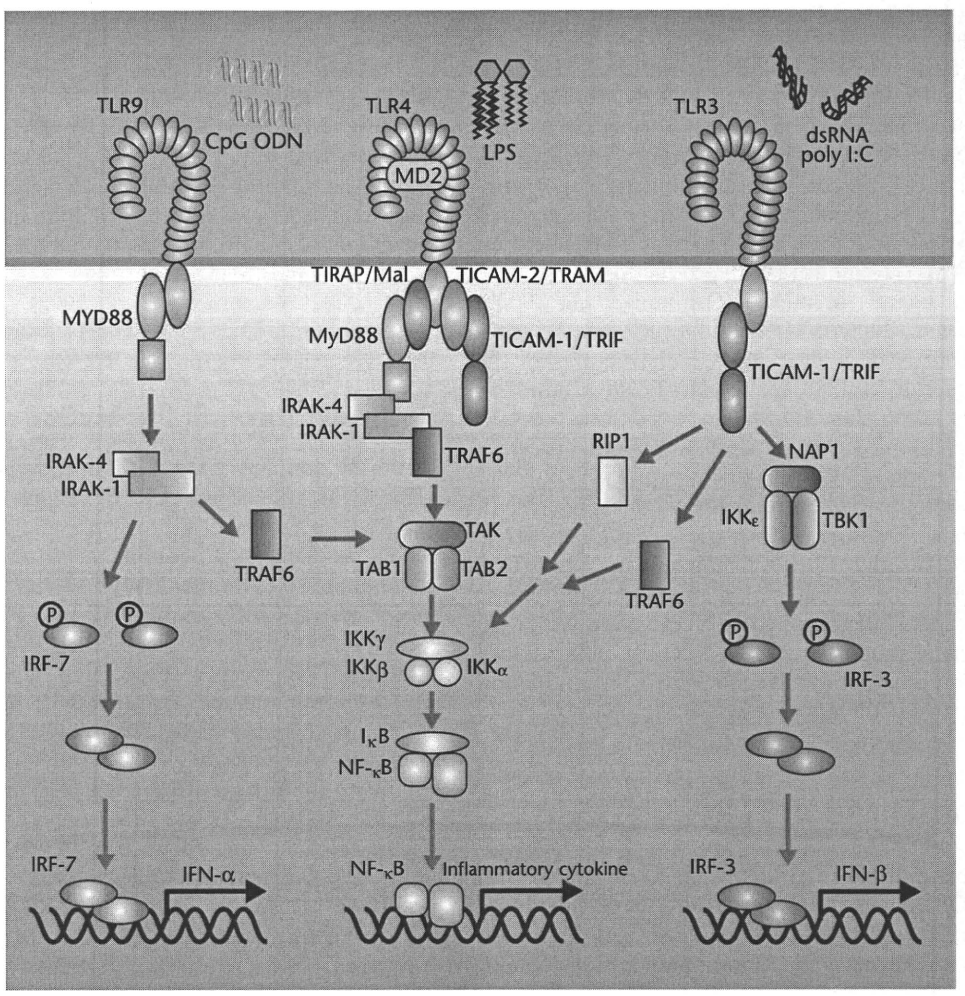
Pathways through the MyD88 adaptor

The TIR domain in the TLR intracytoplasmic region is critical for triggering TLR signalling. Both TLRs and IL1Rs associate with the cytoplasmic adaptor, MyD88, which has a death domain at the *N*-terminal and a TIR domain at the *C*-terminal. MyD88 recruits IL-1R-associated kinase (IRAK) family members through a homotypic interaction between their death domains. Another adaptor, TNF receptor–associated factor 6 (TRAF6), is also recruited and activated through ubiquitination. Ubiquitinated TRAF6 dissociates to bind the TAK1/TAB1/2 complex; subsequently, two major signalling pathways involving the mitogen-activated protein kinase (MAPK) cascades and NF-κB (see Glossary, NF-κB) are activated (**Figure 4a**).

MyD88-deficient mice show abolished responses to IL-1 and IL-18 and to the ligands for TLR2, TLR5, TLR7 and TLR9. TLR4 signalling also requires MyD88 for cytokine induction. Thus, the MyD88-mediated pathway is a



(a)



(b)

common and critical pathway for IL-1R and TLR signalling. NF-κBB activation induced by TLR2 and TLR4 signals is mediated by MyD88 and TIRAP/Mal (Fitzgerald *et al.*, 2001).

Four IRAK family members, that is IRAK-1, IRAK-2, IRAK-M and IRAK-4, have been identified so far. TLR signalling is defective in both IRAK-4-deficient and MyD88-deficient mice. Furthermore, IRAK-4-deficient humans show recurrent bacterial infections, and cells derived from such patients lack TLR responses. IRAK-1 also has serine/threonine kinase activity, but IRAK-1-deficiency does not result in any severe defect in TLR signalling. Instead, IRAK-M-deficient mice exhibit enhanced responses to TLR signalling. Although the phenotype of IRAK-2-deficient mice has not yet been reported, IRAK-4 is regarded as the most critical IRAK for TLR signalling. Consistent with this observation, a MyD88 form that is generated by alternative splicing and can interact with IRAK-1 but not with IRAK-4 acts as a dominant-negative form for TLR-induced NF-κB activation.

Pathways through the TRIF (TICAM-1) adaptor

Studies with MyD88-deficient mice have clarified the diversity of TLR signalling pathways. In MyD88-deficient cells, TLR2, TLR7 and TLR9 signalling fails to induce both NF-κB and MAPK activation. In contrast, TLR4 signalling induces the activation of both NF-κB and IRF-3 (see Glossary, IRF-3), which can lead to IFNβ production (Kawai *et al.*, 2001). Presence of another adaptor was predicted for completing the TLR4 IFNβ-inducing pathway. Meanwhile, it had been reported that TLR3 stimulation induces a unique output of IFNβ induction (Matsumoto *et al.*, 2003). Yeast two-hybrid screening of a human complementary deoxyribonucleic acid (cDNA) library using the TIR sequence of human TLR3 as the bait revealed that TLR3 binds a new TIR-containing adaptor TICAM-1 (Oshiumi *et al.*, 2003a). Overexpressed TICAM-1 per se induces IFNβ, suggesting the importance of this adaptor in selecting the IFNβ-inducing pathway (**Figure 4b**). Ultimately, this adaptor has also been classified as an adaptor for selecting the TLR4 IFNβ-inducing pathway (Oshiumi *et al.*, 2003b). These results were consistent with those obtained from TRIF-knockout (KO) mice (Yamamoto *et al.*, 2003a).

Finally, there are four intracytoplasmic molecules that contain a TIR domain (**Figure 2**). TIRAP/Mal is essential for NF-κB activation downstream of TLR2 and TLR4 (Fitzgerald *et al.*, 2001; Yamamoto *et al.*, 2002). TIRAP/Mal and MyD88 bind to different regions of TLR2 and TLR4 and cooperatively activate NF-κB, which can lead to cytokine gene induction.

IRF-3 activation induced by TLR4 signalling is independent of MyD88 and TIRAP/Mal and instead depends on other adaptors called TRIF (TICAM-1) and TRAM (TICAM-2) (Fitzgerald *et al.*, 2003b; Oshiumi *et al.*, 2003b). TRAM, also called TIRP or TICAM-2, is very similar to TRIF in terms of the amino acid sequences of the TIR domain (**Figure 3**). In TRAM-deficient mice, both the MyD88-dependent and MyD88-independent pathways downstream of TLR4 signalling are defective, but TLR3 signalling is intact (Yamamoto *et al.*, 2003b). TICAM-1 and TRIF are identical according to their structures (Oshiumi *et al.*, 2003a; Fitzgerald *et al.*, 2003a; Yamamoto *et al.*, 2003a). TLR3 signalling also induces IRF-3 activation and IFNβ production through direct coupling with TRIF: TRIF can associate with TLR3 without TRAM, whereas it can associate with TLR4 only in the presence of TRAM (Fitzgerald *et al.*, 2003b; Oshiumi *et al.*, 2003b). Thus, TLR3 signalling is distinct from TLR4 signalling with respect to the requirement for TRAM. The IRF-3-activating pathway by TLR3/4 is greatly impaired in TRIF-deficient cells (Yamamoto *et al.*, 2003a). Thus, TRIF is critically involved in TLR3 and TLR4 signalling.

Two noncanonical IκB kinase (see Glossary, IκB kinase) homologues, IκB kinase ε (IKKε) (also known as inducible IKK (IKKi)) (Fitzgerald *et al.*, 2003b; Sharma *et al.*, 2003) and TRAF family member-associated NF-κB activator (TANK)-binding kinase-I (TBK-I, also known as NF-κB-activating kinase, NAK), are involved in TRIF-induced IRF-3 activation (**Figure 4**). These kinases couple with regulatory molecule, that is NAK-associated protein 1 (NAP1), TANK or SINTBAD, and the complex can

Figure 4 The TLR signalling pathways. (a) MyD88 is the key signalling adaptor for all TLRs with the exception of TLR3 and certain TLR4 signals, IL-1R and IL-18R. Its main role is the activation of NF-κB. It is directly recruited to the TIR domains in certain TLRs (here, shown for TLR5, TLR7, TLR8 and TLR9), and acts to recruit IRAK-4 (IL-1R-associated kinase 4). This leads to a pathway involving IRAK-1, tumour-necrosis-factor-receptor-associated factor 6 (TRAF6), transforming-growth-factor-activated kinase (TAK1) and the ubiquitylating factors ubiquitin-conjugating enzyme E2 variant 1 (UEV1A) and ubiquitin-conjugating enzyme 13 (UBC13), which modify and activate TRAF6 and TAK1. This leads to the activation of the inhibitor of I-κB kinase (IKK) complex and IF-κB and the upstream kinases for p38 and JNK (JUN *N*-terminal kinase). MyD88 is targeted by negative regulators, such as a shorter form known as MyD88s, which interfere with IRAK-4 recruitment, and fatty acid synthase (FAS)-associated via death domain (FADD). IRAK-M can also inhibit signalling by preventing the release of IRAK-1 and IRAK-4 from MyD88. MyD88 also couples to IRF-5 and IRF-1. In the latter case, MyD88 traffics to the nucleus with IRF-1. In the case of TLR2 and TLR4 signalling, a bridging adaptor, MyD88-adaptor-like protein (Mal), is required for MyD88 recruitment. This is subject to regulation by Bruton's tyrosine kinase (BTK) and suppressor of cytokine signalling 1 (SOCS1), which promotes Mal degradation. In the case of signalling by TLR7, TLR8 and TLR9, the MyD88–IRAK-4 pathway also leads through TRAF6 and TRAF3 to the activation of IRF-7. Finally, interferon receptor 1 (IFNR1) can also engage with MyD88 and through mixed-lineage kinase 3 (MLK3) leads to activation of p38. Target genes for each of these pathways are shown. MKK, mitogen-activated protein kinase kinase; TAB, TAK1-binding protein; TNF, tumour necrosis factor; Ub, ubiquitin. (b) IFN-inducing pathway involving TLRs. TLR9 and TLR7 signal exclusively via MyD88 to activate IRF-7 and NF-κB. IRF-7 activation activates the IFNα promoter in pDCs. TLR3 uses TICAM-1 but not MyD88 in mDCs to induce activation of IRF-3 and the IFNβ promoter. TRIF/TICAM-1 recruit RIP1, which together with TRAF6 activates NF-κB. TLR4 signals via both MyD88 and TRIF/TICAM-1 in mDCs. TIRAP/Mal and TRAM/TICAM-2 are adapters involved in coupling TLR4 to MyD88 and TRIF, respectively. Either MyD88 or TRIF promotes activation of NF-κB and MAPKs, leading to transcription of cytokine genes. TRIF in the TLR4 pathway also activates IRF-3, allowing weak IFNβ production.

associate with TRIF and induce phosphorylation and nuclear localization of IRF-3 (Sasai *et al.*, 2006; Ryzhakov and Randow, 2007). Furthermore, IRF-3-dependent gene expression induced by TLR3 and TLR4 signalling is defective in TRAF3-KO cells. Thus, TRIF links TRAF3 for TLR3 and TLR4 signalling with the activation of IKKε/TBK-1 and IRF-3 (Häcker *et al.*, 2006; Oganesyan *et al.*, 2006).

Sterile α motif (SAM) and Armadillo motif (ARM) domain–containing protein (SARM) also carries a TIR domain. SARM has several domains that are possibly involved in protein–protein interactions and plays an essential role in producing antimicrobial peptides in *Caenorhabditis elegans* with Toll-independent signalling. However, in mammals, SARM is not a direct adaptor for TLRs but acts as a negative regulator of the TRIF pathway (Carty *et al.*, 2006).

Other pathways

Regulators of TLR signalling participate in the regulation of the MyD88 and TRIF pathways. Ubiquitination and deubiquitination enzymes also participate in regulation of the TLR pathways. Viral products also control the TLR pathway. Phosphatidylinositol 3-kinase (PI3 kinase) and MAPK also participate in modulation of TLR signalling. It can be assumed that additional molecules, with the exception of TIR-containing adaptors, are required for shaping TLR signalling. Identification of regulatory factors for TLR signalling is in progress.

In conclusion, TLRs play a crucial role in the detection of microbial infections. Further studies are necessary to clarify the precise molecular mechanisms of positive and negative regulations through which TLRs exert their multiple functions.

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Functional evolution of the TICAM-1 pathway for extrinsic RNA sensing

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Summary: The type I interferon (IFN) is a host defense factor against microbial pathogens in vertebrates. In mammals, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) in the cytoplasm are regarded as sensors for double-stranded RNA (dsRNA) and trigger IFN regulatory factor-3 (IRF-3) activation followed by type I IFN induction through the mitochondrial antiviral signaling (MAVS) adapter. This intrinsic pathway appears to link the main protective responses against RNA virus infection in mammals. On the other hand, human Toll-like receptor 3 (TLR3) is localized in the endosomal membrane or cell surface and signals the presence of extrinsic dsRNA. In response to RNA stimulation, TLR3 recruits the Toll-interleukin 1 receptor domain (TIR)-containing adapter molecule 1 (TICAM-1) adapter and induces IRF-3 activation followed by IFN-β promoter activation. Human TLR3 is localized limitedly extent in myeloid dendritic cells, fibroblasts, and epithelial cells. The TICAM-1 and cytoplasmic MAVS pathways converge at the IRF-3-activating kinase in human cells. The reason for the involvement of this extrinsic mode of IFN-inducing pathways in the dsRNA response remains unknown. In fish, two TLRs, i.e. endoplasmic TLR3 and cell surface TLR22, participate in teleost IFN production without the activation of IRF-3. TLR22 is distinct from mammalian TLR3 in terms of cellular localization, ligand selection, and tissue distribution. TLR22 may be a functional substitute for human cell surface TLR3 and may serve as a surveillance molecule for detecting dsRNA virus infection and alerting the immune system for antiviral protection in fish. In this review, we discuss the fundamentals of the extrinsic dsRNA recognition system, which has evolved to induce cellular effectors to cope with dsRNA virus infection across different vertebrate species.

Keywords: *Toll-like receptor, evolution, dsRNA recognition, TICAM-1 (TRIF)*

Introduction

Invading pathogens express specific pattern molecules and are recognized by host pattern recognition receptors (PRRs) (1, 2), representatives of which are Toll-like receptors (TLRs), Nod-like receptors (NLRs), and RNA helicases [retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5), etc.]. These receptors signal the presence of microbial patterns in myeloid dendritic cells (mDCs) and thus induce potent activation of the systemic host defense response (3). Recent studies on pattern receptors of

the innate immune system have increased our understanding of how mDCs mature through infection and subsequently orchestrate cellular immunity (4, 5). These molecules also serve as adjuvants for the induction of antigen-specific acquired immunity. TLRs, RIG-I-like helicases (RLHs), and NLRs are major targets for investigating the induction of robust acquired immune responses upon pathogen stimulation. These studies have been conducted using gene-disrupted mice and in *in vitro* human systems.

It has been reported that human cells induce interferon-β (IFN-β) in response to various RNA structures (6, 7). Double-stranded RNA (dsRNA) and its analog polyinosinic–polycytidylic acid (polyI:C) have been identified as potent immune stimulators of viral patterns and are recognized by PRRs. PRRs link cytoplasmic adapter molecules in these mammalian cells. Cytoplasmic RLH and membrane-associated TLRs that induce IFN-α/β involve the mitochondrial antiviral signaling (MAVS) (also known as IPS-1, Cardif, or VISA) or TICAM-1 [Toll-interleukin-1 receptor (IL-1R) (TIR) domain-containing adapter-inducing IFN-β (TRIF)] adapters, respectively, to converge the signal at IRF-3-activating kinases for IFN-β induction (4, 5, 8). IFN-β induction is IRF-3 dependent in mDCs and fibroblasts/epithelial cells (4, 5). By contrast, IFN-α/β is differentially induced in an IRF-7-dependent manner in plasmacytoid DCs (pDCs) (9). This allows activation of the myeloid differentiation factor 88 (MyD88) adapter protein and IKKα [inhibitor of nuclear factor (NF) κB (IκB) kinase α] kinase, which directly activates the IRF-7 transcription factor (10). However, the molecular assembly and mechanism involved in polyI:C-mediated activation of transcription factors still remain unclear in mice and humans.

Some PRRs preferentially recognize nucleic acid structures that are unique to infectious microbes. Type I IFN induction and cytotoxic T-lymphocyte (CTL)/natural killer (NK) cell activation are major outputs for RNA-sensing PRRs in mammalian cells (5, 11). A variety of RNA sensors in the cytoplasm or membranes are engaged in the detection of microbial RNA. These are expressed in a cell-type specific fashion and participate in IFN-α/β production in various cell types. However, the combinations of these receptors that induce cellular immunity still remain undetermined. It is generally accepted that RNA patterns that are exogenously provided or are produced in bystander cells are internalized by mDCs through phagocytosis and are then recognized by endosomal PRRs. By contrast, RNA patterns produced in the cytoplasm of infected cells are directly recognized by PRRs present in the cytoplasm (12). In this review, we adopted an evolutional approach to study TLRs present on the cell membrane and the recognition of the external dsRNA pattern that is specifically formed in other cells during virus replication.

Fish (teleost) have >20 TLRs that include orthologs of human TLRs and other TLRs unique to lower vertebrates living in water (13, 14). Teleost have orthologs of the IFN-inducing genes of mammals and PRRs for microbial pattern recognition. Teleost also have a TICAM-1 ortholog which has no TRAF-binding site but retains the RIP1-binding site (15, 16). Fish may have orthologs of RLH and NLRs. Hence, by comparing the mammalian PRR receptor/adapter system with that of fish, it is possible to examine the development of the innate recognition system during evolution. Molecular evolution by which the mammalian immune system has been established in the current form can be analyzed through the genomic information of vertebrate TLR systems. In this study, we cast insight into the functional properties of fish TLRs and adapters involved in IFN induction.

Recognition of RNA duplexes in vertebrates

Viral replication usually generates dsRNA in the cytoplasm of infected cells and signals to activate antiviral responses. dsRNA, stem-loop structure of RNA, 5′-uncapped triphosphate of RNA, and specific RNA sequences are rapidly recognized by PRRs in the cytoplasm (4, 5, 17), then implicated in host defense (Fig. 1). Many pattern-sensing receptors have been identified in mammals: PKR (dsRNA-dependent protein kinase), Dicer of the short interfering/microRNA system, RLHs including RIG-I, MDA5, and LGP2, and other helicases. These receptors are accompanied by adapters that transduce the dsRNA-sensing signal downstream. Other RNA-sensing molecules such as helicases may also be present in the cytoplasm to join a molecular assembly for foreign RNA detection. The synthesized dsRNAs are incorporated into these molecular complexes to prohibit RNA replication in virus-infected cells.

TLR3 is present in the early endosome and can recognize dsRNA delivered inside the endosomal membrane (18). TLR3 may not have a direct role in capturing dsRNA generated by virus replication in the cytoplasm, but it has an important role in trapping phagocytosed dsRNA (Fig. 2), which is usually wrapped in a membrane that originates from the infected cell (19). In comparison to the direct recognition system of dsRNA in the cytoplasm, this mode of RNA recognition is unique and sophisticated, concerning activation of cellular immunity. As RNA-sensing TLRs and RLH are conserved across vertebrates (20), we hypothesize

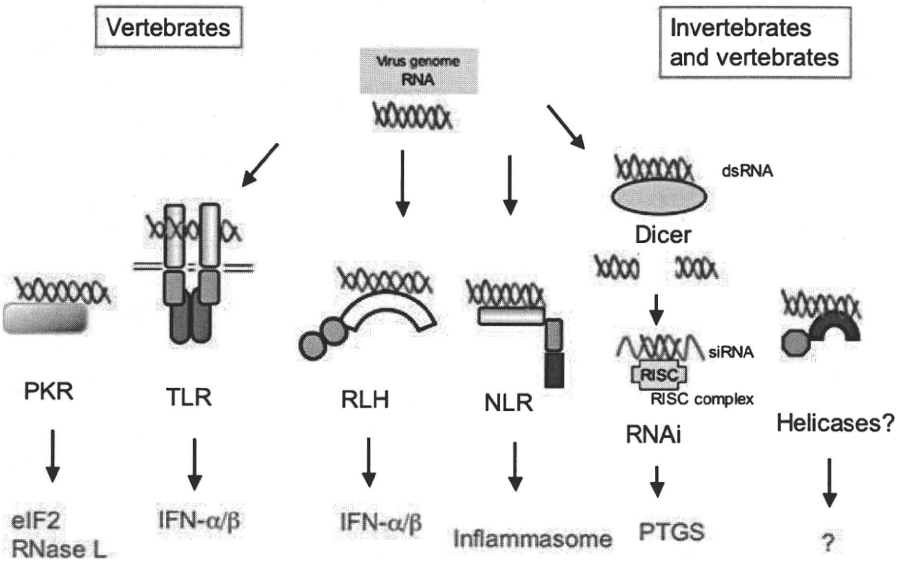

Fig. 1. Various RNA sensors in vertebrates. dsRNA are generated during virus replication. Major RNA sensors in vertebrate cells and their responses on stimulation with dsRNA are indicated. Dicer and RNA-recognizing helicases work even in invertebrates. How dsRNA selects a variety of RNA pattern sensors remains largely unknown. PTGS, post-transcriptional gene silencing.

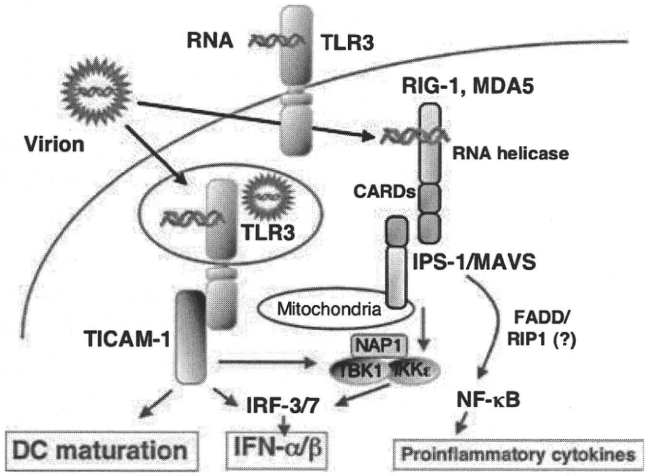

Fig. 2. Cell surface, endosomal and cytoplasmic recognition of dsRNA in mammalian cells. TLR3 is distributed either on the cell surface or in the endosome. Its distribution ratio depends on cell types. RLH (RIG-I and MDA5) reside in the cytoplasm. Adapter molecules, TICAM-1 and MAVS, are localized in the cytoplasm. Upon stimulation, TLR3 recruits TICAM-1 near the endosomal membrane, while MAVS recruits RLH on the mitochondrial membrane. The known outputs of TLR3 and RLH are indicated by red. TLR, Toll-like receptor; RIG, retinoic acid-inducible gene; RLH, RIG-I-like helicase; TICAM, Toll-interleukin 1 receptor domain-containing adapter molecule.

that the two distinct pathways of dsRNA recognition were established in a vertebrate ancestor ~500 Ma and that the two systems have been preserved in mammals (Fig. 2). We focus on the fish membrane-associated dsRNA recognition system and analyze it in terms of its physiological significance and functional feature and also from an evolutionary point of view. We also address the question of why vertebrates need the surface system for dsRNA recognition in addition to the cytoplasmic virus-sensing systems.

Surface recognition of dsRNA in mammals

We initiated a study on the functions of the membrane-associated dsRNA recognition receptor TLR3 in human cells. Stimulation of human fibroblasts/epithelial cells with polyI:C leads to the production of type I IFN. We have produced monoclonal antibodies (mAbs) against human TLR3 and obtained one which blocks polyI:C binding to TLR3, named the mAb TLR3.7 (21). The TLR3.7 mAb interferes with IFN-β production induced by exogenously added polyI:C in human fibroblasts/epithelial cells (18, 21). Hence, it appears that TLR3.7 mAb blocks the interaction between TLR3 and polyI:C on the cell surface by binding to TLR3. If this is the case, human TLR3 must be localized on the cell surface of the fibroblast to capture external dsRNA. This hypothesis was proven by results from fluorescence-assisted cell sorting (FACS) and imaging analyses (Fig. 3A). However, using the same mAb, human mDC TLR3 could not be detected on the surface (18) but was found to be localized in intracellular compartments, particularly endosome (Fig. 3A). mDCs respond to polyI:C to induce type I IFN in the early endosome (22, 23). In this case, how does endosomal TLR3 recognize polyI:C outside the cells? It is rational that there is a transporter that shuttles dsRNA from the cell surface to the endosome in mammals (5). The recognition of dsRNA by TLR3 on the cell surface is

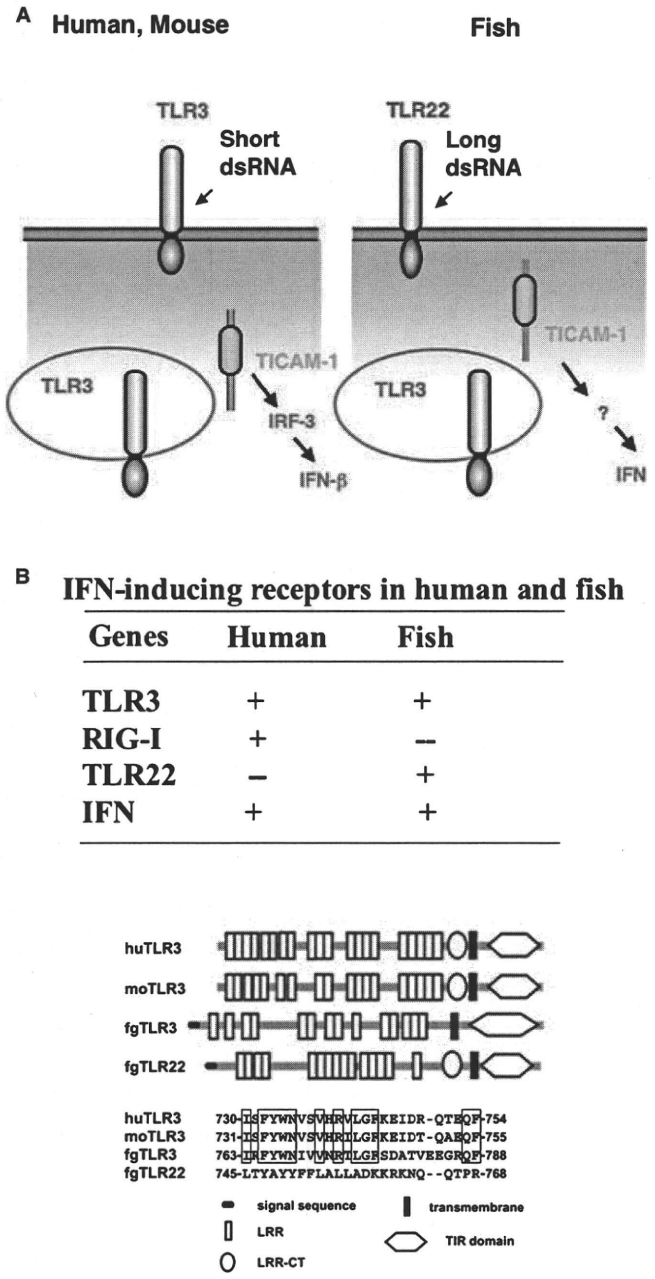

Fig. 3. Different TLRs cover surface dsRNA recognition in fish and mammals. (A) TLR3 and TLR22 in vertebrates. In human and mouse, TLR3 encompasses cell surface and endosomal RNA sensing and induces IRF-3 activation. In fish, two distinct gene products, TLR3 and TLR22, participate in dsRNA sensing. IFN is induced in an IRF-3-independent fashion. Although the structural information is not shown in the panel, mammalian TICAM-1 structurally differs from fish TICAM-1. IRF-3-activating kinase indirectly assembles in an N-terminal portion of mammalian TICAM-1 but not fish TICAM-1. A C-terminal portion contributes to IFN promoter activation in fish cells. (B). Difference of IFN-inducing receptors between human and fish. Upper table indicates that humans lack TLR22 while fish lack RIG-I, although both have IFN-inducible pathways. The structural differences among human (hu) TLR3, mouse (mo) TLR3, fgTLR3, and fgTLR22 are depicted in the lower panel. The primary structures of the linker region (a determinant of TLR3 localization) are shown below the structural models.

experimentally proven by using the mAb probe for determining the localization of human TLR3. However, the dsRNA shuttling system has not yet been proved.

If TLR3 participates in the induction of IFN-β in epithelial cells, its downstream molecules should activate IRF-3. Therefore, we searched for an adapter molecule that could directly interact with TLR3 and activate IRF-3; the molecule was identified by employing the yeast two-hybrid system. It was named TICAM-1 (24) and is now popularly known as TRIF (25).

Human TICAM-1 consists of an N-terminal region (1–234), a TIR domain (235–500), and a C-terminal region (501–680). The N-terminal region of TICAM-1 harbors tumor necrosis factor (TNF) receptor-associated factor (TRAF) family proteins (26, 27) and forms a complex containing IRF-3-activating kinases (28, 29). This kinase complex is crucial for activating the IFN-β promoter (28, 29) and inducing the activation of NK (5, 30) and CTL (12, 31) effector cells (*Fig.* 4). The C-terminal region of TICAM-1 can recruit receptor-interacting protein-1 (RIP-1), and this event is followed by the activation of other effectors (32). All these signaling events constitute the TICAM-1 pathway. Human and mouse TICAM-1 pathways involve mDC maturation, cytokine/chemokine induction, cross-presentation of exogenous antigens for proliferation of $CD8^+$ T cells (5, 12, 31, 33), NK cell activation (30, 34), and induction of autophagy and apoptosis (35). CD4+ regulatory T (Treg) cells and Th17 cells may be induced by mDCs matured through TICAM-1 signaling. TICAM-1 may act as a platform that recruits various signaling molecules for mDC output in mammals. However, one question that remains unanswered is whether the TICAM-1 pathway is conserved in lower vertebrates such as fish.

Surface recognition of dsRNA in fish

Fish [*Takifugu rubripes* (fg)] have ~20 TLRs and three TLR adapters, i.e. fgMyD88, fgTICAM-1, and fgTIRAP/Mal (36). By using the yeast two-hybrid analysis system, we found at least two TLRs that share the fgTICAM-1 adapter (37). The first report on fgTLRs (13) showed that fgTLR3 and fgTLR22 choose the fgTICAM-1 adapter in fish cells and induce fish type I IFN by recognizing dsRNA. fgTLR3 and fgTLR22 are quite different in their primary structures (*Fig.* 3B) and are classified into different clades by gene tree analysis (13, 37). However, both fgTLR3 and fgTLR22 directly bind to fgTICAM-1 in fish cells as well as in yeast. Confocal analysis has shown that fgTLR3 resides in the endoplasmic reticulum (ER) and recognizes relatively short dsRNA, whereas fgTLR22 recognizes long dsRNA present on the cell surface (37). The

properties of fgTLR3 and fgTLR22 are summarized in Fig. 3B. fgTLR22 is particular, as fgTLR22 preferentially recognizes long dsRNA, localizes exclusively to the cell surface, and is widely distributed across tissue/organs. In summary, two of the receptors that recognize dsRNA are also involved in the TICAM-1 pathway in fish. The fish TICAM-1 pathway leads to the activation of the IFN promoter.

The next question is how TICAM-1 is assembled by TLR22 to transmit the dsRNA recognition signal. Possible answers may lie in the structural difference between mammalian and teleost TICAM-1 (Fig. 3B). Over-expression of zebrafish (zf)TICAM-1 activates the zfIFN promoter, but zfTICAM-1 does not interact with zfTRAF6 (16). Results from genomic retrieval analysis suggest that zebrafish lacks IRF-3. The zfTICAM-1 N-terminal region does not contain the TRAF6-binding motif (that participates in IRF-3 activation), and the C-terminal region of zfTICAM-1 can adequately activate the zfIFN promoter. This observation suggests the involvement of RIP1-mediated NF-κB activation in zfIFN promoter activation (16, 37).

Human TICAM-1 stimulates IRF-3-mediated type I IFN induction by means of its N-terminal region (38, 39) (Fig. 4). Thus, fish TICAM-1 behaves like human TICAM-1; however, fish TICAM-1 does not employ IRF-3 to activate the IFN-β promoter (16, 40). Although the TICAM-1 pathway is conserved across both fish and humans, the molecular bases for IFN induction in response to extrinsic dsRNA differ in the two vertebrate species (Fig. 3). Our speculation is that although fish cells have an IFN output similar to that of human cells, the signal cascade that leads to IFN production is modally different. Teleost TICAM-1, which is structurally dissimilar to human TICAM-1 (36), might help in explaining the differential selection of the signal pathways.

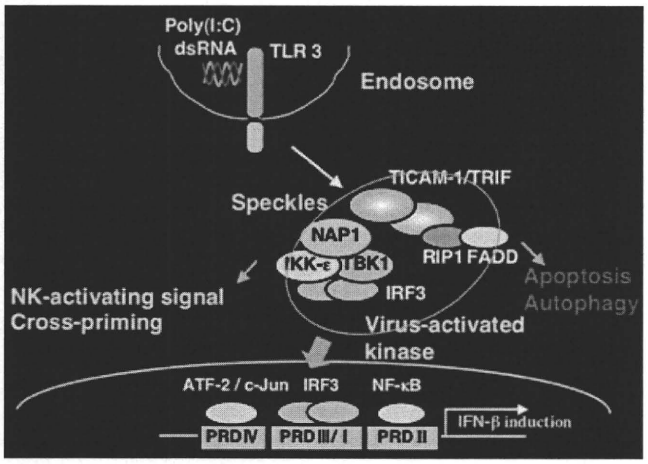

Fig. 4. TICAM-1 is dissociated from TLR3 to form a signaling unit, Speckle. In human cells, TICAM-1 once detached from TLR3 serves as a signaling platform to induce apoptosis, autophagy, NK activation, and cross-priming. TICAM-1 undergoes some modification secondary to complex formation with TLR3 and dissociated from TLR3 with unknown mechanism. The pathways for NK activation, CTL induction, and autophagy are not yet identified, although the pathway for apoptosis is getting clarified. It is undetermined whether surface-expressed TLR3 or TLR22 retain the cellular responses.

How does human TLR3 substitute for TLR22 in mammals?

The differences between TLR22 and TLR3 can be summarized as follows. Based on confocal microscopy and FACS analyses, over-expressed fgTLR22 is localized on the cell surface, while fgTLR3 resides in the ER and endosomes in fish cells (37). fgTLR22 is ubiquitously distributed over the organs/tissues of teleost, while human and fgTLR3 are present only in a limited cell repertoire. These two TLRs do not merge with each other or with fgTICAM-1 in resting cells. When stimulated with polyI:C, a part of the fgTLR22 population enters the cytoplasmic region to merge with fgTICAM-1 (37). Similarly upon stimulation, fgTLR3 is clustered and merges with fgTICAM-1 in the cytoplasm (37). Immunoprecipitation studies have supported their molecular interactions: fgTICAM-1 coprecipitates with fgTLR22 or fgTLR3 in human HEK293 cells. A reporter assay has shown that the dominant-negative form of fgTICAM-1 blocks the fgTLR22- and fgTLR3-mediated IFN promoter activation induced by endogenous fgTICAM-1 in RTG-2 (rainbow trout) cells. Thus, fish have a novel TICAM-1-coupling TLR, TLR22, which is clustered on the cell surface. Although mammals have lost TLR22, TLR3 is distributed on the surface membrane as well as in the endosomes only in some kinds of epithelial cells (41–44), and this appears as though TLR3 compensates for the loss of TLR22 in limited cell types.

We tested the physiological function of fgTLR22 and found that fgTLR22-expressing RTG-2 (rainbow trout) cells become resistant to virus infection (37). We used birnavirus, which is a representative dsRNA virus found in water. Cytopathic effect formation was observed in control cells that did not express fgTLR22, whereas it was barely detected in cells expressing fgTLR22. The level of TCID50 in the supernatant, which reflects virus replication in the cells, was high in the control cells and ~100-fold lower in fgTLR22-expressing cells. Virus RNA levels were suppressed in fgTLR22-expressing cells. Conversely, IFN mRNA was upregulated in virus-infected cells.

In humans, TLR3 is expressed in the endosomes and on the surface of epithelial cells/fibroblasts (18, 22). Expression of TLR3 on the cell surface membrane of human bronchial, bile-duct, and intestinal epithelial cells has also been reported

(41–44). Thus, surface-expressed human TLR3 appears to be a functional remnant of fish TLR22: TLR3 functions in the mucosal region wherein body fluids are continuously in contact with the flora. Because cell surface-associated dsRNA recognition is indispensable even in humans, TLR3 is expressed on human fibroblasts and epithelial cells. Likewise, TLR22 may be a functional substitute for human cell surface TLR3 and may act as a surveillance molecule for detecting dsRNA virus infection.

Evolution of the surface RNA recognition system in vertebrates

The results from bootstrap probability analysis indicate that TLR22 does not belong to the TLR3 family and is instead proximal to mouse TLR13, which has not been characterized as a dsRNA-recognizing TLR. Thus, two arms of the TICAM-1 pathway have evolved as dsRNA receptors in fish, and only TLR3 has been preserved in mammals (*Table* 1). Development of TLR22 instead of TLR3 may afford some advantage for protection against RNA viruses by augmenting the susceptibility of the local IFN response to long RNA duplexes.

We wanted to understand why teleosts require a cell surface RNA recognition system. Fish live in water and are exposed to many kinds of negative-stranded RNA viruses belonging to the Rhabdoviridae and dsRNA viruses (45, 46). Bacteria such as *Rhodovulum sulfidophilum* and perhaps other species are involved in the extracellular liberation of ribosomal and transfer RNAs into the sea (47). Thus, the sea may contain RNA viruses and RNA products of microbial origin. The sea is home to a unique and mysterious microbial environment. During evolution, vertebrates in water may have been protected from these pathogens by developing a set of RNA-sensing TLRs and an IFN system, which are distinct from those expressed in land animals. Our studies indicate that RNA sensing by TLRs protects fish from spreading or exacerbating infection. Land animals preserve the surface RNA recognition system to a limited extent in their epithelial ducts where the microbial environment is retained similar to that found in the sea.

Over-expressed teleost TLR22 protects host cells from infection with IPNV, which is a naked bisegmented dsRNA virus belonging to the family Birnaviridae (48). Birnaviruses have a single T = 13 *icosahedral* shell that is composed of 120 subunits, and these viruses lack the characteristic inner capsid. Aquatic birnaviruses are distributed worldwide, can infect a range of fish and shellfish species (45, 46), and are viral pathogens that cause diseases in fry and young fish. Although teleosts have the gene that encodes a putative ortholog of the cytoplasmic RNA sensor MDA5 (36, 49), IPNV efficiently infects teleost cells unless TLR22 is expressed in some population of cells. Thus, fish MDA5 is insufficient for protection against this type of dsRNA virus. Although all cells do not express TLR22, IFN seems to be sufficiently induced by TLR22-expressing cells to provide an antiviral environment in surrounding cells, resulting in host cell protection. However, the manner in which TLR22 detects the IPNV infection remains to be clarified. The necessity of TLR22 and its mode of dsRNA recognition in fish are of interest for further investigation.

Effector induction by endosomal TLR3 in mammals

We produced a TICAM-1 knockout (KO) mouse and tested the effector-inducing properties using the syngeneic tumor implant system of this mouse (30, 50). PolyI:C was intraperitoneally administered as the ligand for TLR3 stimulation. In this system, RLH may sense polyI:C similarly in TICAM-1 KO as well as in wildtype mice, but detectable phenotypes shouldreflect only the difference in TICAM-1 in mice. Mouse melanoma line B16

Table 1. Repertoire of pattern recognition receptors in vertebrates

	TLR																			
	1	2	3*	4	5	6	7	8	9	10	12	13	14	21	22*	MyD88	TICAM*	RIG-I	MDA-5	IPS-1	IFN
Human	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+
Mouse	+	+	+	+	+	+	+	+	+	*psd*	+[†]	+	–	–	–	+	+	+	+	+	+
Chicken	+	+	+	+	+	*psd*	+	–	–	–	–	–	+[‡]	+	–	+	+	–	+	*frg*	+
Xenopus	+	+	+	+	+	±	+	+	+	±	+	+	+	+	+	+	+	+	+	*frg*	+
Fugu	+	+	+	–	+	–	+	+	+	–	–	–	+	+	+	+	+	–	+	*frg*	+
Zebra	+	+	+	+	*frg*	–	*frg*	*frg*	+	–	+	–	+	*frg*	+	+	+	–	+	*frg*	+
Ascidia	~3?															–	–	–	–	–	–
Sea urchin	~300?															7	2	6?	6?	1?	–

psd, pseudogene; *frg*, fragment.
*TLR3, TLR22, and TICAM are IFN-inducing genes.
[†]Mouse TLR11.
[‡]Bird TLR15.
Ascidia and Sea urchin are invertebrate references.

[low major histocompatibility complex (MHC) expresser] and the C57BL/6 cell lines were used in this study.

The tumors grew well in wildtype mice. When polyI:C was administered intraperitoneally, tumor growth was retarded. Similar results were obtained with MyD88 KO, PKR KO, and IFN-β KO mice. PolyI:C-mediated tumor growth retardation was completely abrogated in TICAM-1 KO mice, suggesting that TICAM-1 is crucial for tumor-directed effector induction. IFN-β is an output of the activation of the TICAM-1 pathway, but it barely affects tumor regression. Retardation of tumor growth by polyI:C was completely abrogated in wildtype mice by depletion of NK1.1- or asialoGM-1-positive cells (30). Tumor growth suppression in response to polyI:C was normally observed in $CD8^+$ T-cell-depleted mice. Hence, NK/NKT cells, not CTLs, are effectors responsible for tumor regression in this mouse model with low MHC-expressing tumor. As polyI:C activates the TICAM-1 pathway, size reduction of the implant tumor reflects the potential of the effectors induced by the functioning of the TICAM-1 pathway (Fig. 5).

We next checked whether TICAM-1 in mDCs or other immune cells is important for tumor growth retardation. TICAM-1 was transfected into bone marrow-derived DCs (BMDCs), and these cells were adoptively transferred to mice with tumor burden. Tumor growth was significantly reduced in mice injected with TICAM-1-positive BMDCs but not in those injected with other BMDCs that did not express TICAM-1 (50). Thus, the mDC TICAM-1 pathway is involved in anti-tumor NK activation (30) (Fig. 5).

The TICAM-1 pathway activates transcription factors, IFN regulatory factor-3 (IRF-3), IRF-7, activator protein 1 (AP1), and NF-κB in mouse cells. The results from our *in vitro* NK assay suggest that IRF-3 largely participates in mDC-NK reciprocal activation (T. Ebihara, M. Matsumoto, T. Seya, unpublished data). Actually, polyI:C-mediated tumor growth retardation was abrogated in IRF-3 KO mice but not IRF-7 KO mice. Thus, in mDCs, induction of the molecules that drive NK activation would depend on IRF-3 activation.

We found that tumor-specific CTLs are induced by polyI:C when EG7 cells [a high MHC expresser with ovalbumin (OVA)] are employed as the implant tumor. Therefore, we checked the levels of the OVA epitope-responsive $CD8^+$ T cells, i.e. OT-1. BMDCs expressing TICAM-1 potentially induce T-cell proliferation and IFN-γ induction (Fig. 4). These T-cell responses are largely independent of IRF-3 or IRF-7 in mDCs (M. Azuma, T. Ebihara, M. Matsumoto, T. Seya, unpublished data). Thus, when implant tumor expresses high levels of MHC, CTLs driven through mDCs act as the main effector cell in mice (31). CTLs and NK cells are induced by distinct routes in mDCs (51, 52).

Cellular immune activation by mDCs depends on the situation of TLR3-adapter complex. Cytoplasmic activation of the

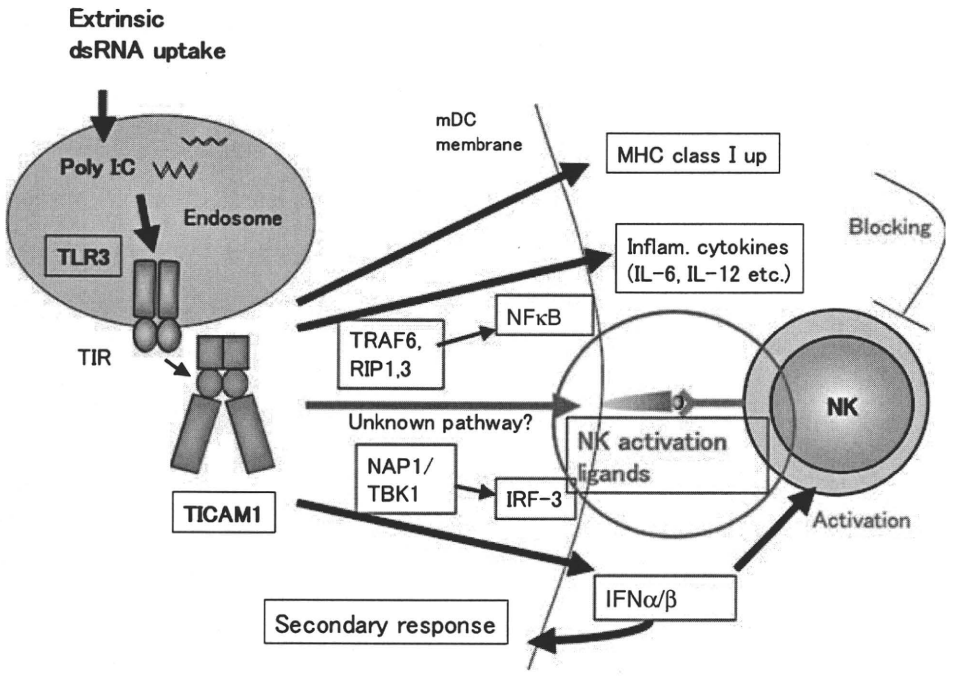

Fig. 5. Mechanism of mDC-NK reciprocal activation induced by dsRNA stimulation of mDCs. TICAM-1 has a crucial role in NK activation driven by polyI:C-stimulated mDCs in human cells. When TLR3 grasps the dsRNA signature in the endosome of mDCs, TICAM-1 in mDCs is activated to evoke a signal pathway reaching to the expression of NK-activating ligands. NK cell activation is then induced via mDC-NK contact. Some soluble factors may be important for NK activation in addition to the expression of NK-activating ligands.

