

Figure 10. Model for Riplet-mediated RIG-I activation. In resting cell, RIG-I RD represses its CARDs-mediated signaling. When RIG-I CTD associates with viral RNA, Riplet mediates K63-linked polyubiquitination of RIG-I RD, leading to the association with TRIM25 and TBK1. K63-linked polyubiquitin chain mediated by TRIM25 induces RIG-I oligomerization and association with IPS-1 adaptor. TBK1 associated with RIG-I is activated on mitochondria.

doi:10.1371/journal.ppat.1003533.g010

pEF-BOS/FLAG-RIG-I plasmid as a template. The primer sequences are RIG-I 849 851 RR-F: AGT AGA CCA CAT CCC AGG CCA AGG CAG TTT TCA AGT TTT G, RIG-I 849 851 RR-R: CAA AAC TTG AAA ACT GCC TTG GCC TGG GAT GTG GTC TAC T, RIG-I 888R-F: GAC ATT TGA GAT TCC AGT TAT AAG AAT TGA AAG TTT TGT GGT GGA GG, RIG-I 888R-R: CCT CCA CCA CAA AAC TTT CAA TTC TTA TAA CTG GAA TCT CAA ATG TC, RIG-I 907 909RR-F: GTT CAG ACA CTG TAC TCG AGG TGG AGG GAC TTT CAT TTT GAG AAG, RIG-I 907 909RR-R: CTT CTC AAA ATG AAA GTC CCT CCA CCT CGA GTA CAG TGT CTG AAC. HCV cDNA fragment encoding NS3-4A of

JFH1 strain was cloned into pCDNA3.1 (-) vector. The mutation on catalytic site of NS3-4A S139A was constructed by PCR-mediated mutagenesis using primers, NS3-4A S139A-F and NS3-4A S139A-R, and pCDNA3.1 (-)/NS3-4A plasmid as a template. The primer sequences are NS3-4A S139A-F: TTC GAC CTT GAA GGG GTC CGC GGG GGG ACC GGT GCT TTG C and NS3-4A S139A-R: AAG CAC CGG TCC CCC CGC GGA CCC CTT CAA GGT CGA AAG G.

RT-PCR and Real-Time PCR

Total RNA was extracted with TRIZOL (Invitrogen), after which the samples were treated with DNaseI to remove DNA

contamination. Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (ABI). Quantitative PCR analysis was performed using Step One software v2.0. (ABI) with SYBER Green Master Mix (ABI). HCV ss and dsRNA was in vitro synthesized with SP6 and/or T7 RNA polymerase using 3' UTR of HCV cDNA as template as described previously [46].

Confocal microscopy

Cells were plated onto microscope cover glasses (matsunami) in a 24-well plate. The cells were fixed for 30 min using 3% formaldehyde in PBS and permeabilized with 0.2% Triton X-100 for 15 min. Fixed cells were blocked with 1% bovine serum albumin in PBS for 10 min and labeled with the indicated primary Abs for 60 min at room temperature. Alexa-conjugated secondary Abs were incubated for 30 min at room temperature to visualize staining of the primary Ab staining. Samples were mounted on glass slides using Prolong Gold (Invitrogen). Cells were visualized at a magnification of $\times 63$ with an LSM510 META microscope (Zeiss). Data collected with confocal microscopy were analyzed with ZEISS LSM Image Examiner software. NS3, RIG-I, TBK1, IPS-1, and p-TBK1 were stained with anti-NS3 goat pAb (abcam), anti-RIG-I mouse mAb (Alme-1, ALEXIS BIOCHEMICALS), anti-NAK (TBK1) rabbit mAb (EP611Y, abcam), anti-MAVS (IPS-1) rabbit pAb (Bethyl Laboratories Inc), and anti-p-TBK1 rabbit mAb (Cell Signaling Technology),

Reporter gene analysis

HEK293 cells were transiently transfected in 24-well plates using FuGene HD (Promega) or lipofectamine 2000 (Invitrogen) with expression vectors, reporter plasmids (IFN- β : p125luc), and an internal control plasmid coding *Renilla* luciferase. The total amounts of plasmids were normalized using an empty vector. Cells were lysed in a lysis buffer (Promega), and luciferase and *Renilla* luciferase activities were determined using a dual luciferase assay kit (Promega). Relative luciferase activities were calculated by normalizing the luciferase activity by control. HCV dsRNA (3' UTR polyU/UC region) was synthesized using T7 and SP6 RNA polymerase as described previously [46].

Pull-down assay

RNA used for the assay was purchased from JBioS. The RNA sequences are as follows: (sense strand) AAA CUG AAA GGG AGA AGU GAA AGU G; and (antisense strand) CAC UUU CAC UUC UCC CUU UCA GUU U. Biotin was conjugated at the U residue at the 3'-end of the antisense strand (underlined). Biotinylated dsRNA was phosphorylated by T4 polynucleotide kinase (TAKARA). dsRNA was incubated for one hour at 25°C with 10 μ g of protein from the cytoplasmic fraction of cells that were transfected with Flag-tagged RIG-I, Riplet, and/or HA-tagged ubiquitin expressing vectors. This mixture was added into 400 μ l of lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% Glycerol, 1% NP-40, 30 mM NaF, 5 mM Na₃VO₄, 20 mM iodoacetamide, and 2 mM PMSF) containing 25 μ l of streptavidin Sepharose beads, rocked at 4°C for two hours, harvested by centrifugation, washed three times with lysis buffer, and resuspended in SDS sample buffer.

Immunoprecipitation

Splenocytes (1×10^7) were infected with or without VSV at MOI = 10 for eight hours, after which cell extracts were prepared with lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 30 mM NaF, 5 mM

Na₃VO₄, 20 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride). Immunoprecipitation used an anti-RIG-I Rabbit monoclonal antibody (D14G6, Cell Signaling Technology). To detect endogenous K63-linked polyubiquitin chain that is ligated to RIG-I, 6×10^7 of mouse splenocyte were infected with SeV at MOI = 0.2 for 24 hours. Immunoprecipitation was performed with anti-RIG-I mAb (D14G6). Anti-K63-linkage specific polyubiquitin (D7A11) Rabbit mAb (Cell Signaling) was used for western blotting. HEK293FT cells were transfected with or without 0.8 μ g of HCV dsRNA in a 6-well plate. HCV dsRNA (HCV 3' UTR polyU/UC region) was synthesized using T7 and SP6 RNA polymerase as previously described [46]. Cell lysates were prepared at the indicated times. Immunoprecipitation was performed with an anti-RIG-I mouse monoclonal antibody (Alme-1). An anti-FLAG M2 monoclonal antibody (Sigma) was used for the immunoprecipitation of FLAG-tagged protein. An anti-TRIM25 rabbit polyclonal antibody (abcam), an anti-p-TBK1 rabbit mAb (Cell Signaling Technology), an anti-NAK (TBK1) rabbit mAb (EP611Y), and an anti-RNF135 (Riplet) pAb (SIGMA), were used for western blotting. For ubiquitination assay, immunoprecipitates were washed three times with high salt lysis buffer ((20 mM Tris-HCl pH 7.5, 1M NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 30 mM NaF, 5 mM Na₃VO₄, 20 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride) to dissociate unanchored polyubiquitin chain [21], and then washed once with normal lysis buffer described above for SDS-PAGE analysis. Band intensity was semi-quantified using Photoshop software.

RNAi

siRNAs for human Riplet (Silencer Select Validated siRNA) and negative control were purchased from Ambion. siRNA sequences for Riplet are: (sense) GGA ACA UCU UGU AGA CAU Utt and (anti-sense) AAU GUC UAC AAG AUG UUC CCac. siRNA was transfected into cells using RNAiMax Reagent (Invitrogen) according to the manufacture's instructions.

In vitro NS3/4A cleavage assay

FLAG-tagged Riplet was expressed in HEK293FT cells, and cell lysate was prepared with the lysis buffer described above. The protein was immunoprecipitated with anti-FLAG antibody and protein G sepharose beads, and washed with Buffer B (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40). The samples were suspended in 50 μ l of Buffer B, and incubated with 400 ng of recombinant NS3-4A (rNS3-4A) protein at 37°C for one hour, and then subjected to SDS-PAGE analysis. The NS3-4A protein was purchased from AnaSpec Inc (CA). N-terminal GST-fused Riplet (1–210 aa) (rRiplet) was purchased from Abnova. 500 ng of rRiplet was incubated with or without 500 ng of rNS3-4A in 10 μ l of reaction buffer (20 mM Tris-HCl (7.5), 4% Glycerol, 5 mM DTT, 150 mM NaCl, 0.1% of Triton-X100, 0.9% polyvinyl alcohol) at 37°C for 30 min.

Accession numbers

The accession numbers are Riplet (BAG84604), TRIM25 (NP_005073), TBK1 (NP_037386), IKK- ϵ (AAF45307), IPS-1 (BAE79738), RIG-I (NP_055129), and G3BP (CAG38772).

Supporting Information

Figure S1 K63-linked polyubiquitination of RIG-I RD. HA-tagged ubiquitin and FLAG-tagged RIG-I RD expression vectors were transfected into HEK293FT cells. 24 hours after transfection, the cells were infected with VSV at MOI = 1 for six

hours. Then, cell lysate was prepared. Immunoprecipitation was carried out using anti-FLAG antibody. The samples were subjected to SDS-PAGE, and the proteins were detected by western blotting using anti-HA, FLAG, and K63-linked polyubiquitin specific antibodies.
(TIF)

Figure S2 Intracellular localization of RIG-I, NEMO, and p-TBK1 proteins. (A) HeLa cells were transfected with HCV dsRNA using lipofectamine 2000 reagent. The cells were fixed six hours after transfection. The microscopic analysis was performed using anti-RIG-I mAb (Alme-1) and anti-NEMO pAb. (B) HeLa cells were transfected with HCV dsRNA using lipofectamine 2000 reagent (Invitrogen). The cells were fixed at indicated hour. The microscopic analysis was performed using anti-RIG-I mAb (Alme-1). (C) HepG2 cells were transfected with HCV dsRNA using lipofectamine 200 reagent. The cells were fixed six hours after the transfection. The microscopic analysis was performed using anti-RIG-I (Alme-1) mAb and anti-p-TBK1 mAb.
(TIF)

Figure S3 NS3-4A of HCV cleaves IPS-1 and Riplet but not IKK-ε. (A) HA-tagged Riplet was transfected into HEK293 cells together with NS3-4A. 24 hours after transfection, cell lysate was prepared and subjected to SDS-PAGE. The proteins were detected by western blotting and CBB staining. (B, C) HA-tagged IKK-ε (B) or IPS-1 (C) expression vectors were transfected into HEK293FT cells with or without NS3-4A of HCV expression vector. 24 hours after the transfection, the cell lysate was prepared, and analyzed by SDS-PAGE. The proteins were detected by western blotting using anti-HA or anti-β actin antibodies. (D) HA-tagged IPS-1 or HA-tagged Riplet expression vector was transfected into HEK293FT cells with or without NS3-4A expression vectors. 24 hours after transfection, cell lysate was prepared and subjected to SDS-PAGE. The proteins were detected by western blotting using

anti-HA antibody. (E, F) N-terminal FLAG-tagged Riplet (E) or C-terminal HA-tagged Riplet (F) expression vector was transfected into HEK293FT cells with NS3-4A or NS3-4A*. 24 hours after the transfection, cell lysates were analyzed by SDS-PAGE. (G) HA-tagged wild-type Riplet or mutant Riplet-C21A expression vector were transfected into HEK293FT cells with NS3-4A or NS3-4A*. 24 hours after the transfection, the cell lysate was prepared, and analyzed by SDS-PAGE. The proteins were detected by western blotting using anti-HA or anti-β actin antibodies. (H, I) RIG-I, Riplet, Riplet-3A (H), and/or Riplet C21A (I) mutant expression vectors were transfected into HEK293 cells together with p125luc reporter and *Renilla* luciferase. 24 hours after transfection, luciferase activity was measured.
(TIF)

Figure S4 siRNA for Riplet or control was transfected into HeLa cells in 24-well plate using RNAi MAX (Invitrogen) according to manufacture's protocol. 48 hours after transfection, the cells were transfected with 100 ng of HCV dsRNA. Six hours after transfection, the cells were fixed and stained with anti-RIG-I mAb (Alme-1) and anti-mouse Alexa-488 Ab.
(TIF)

Acknowledgments

We thank Dr. Shimotohno K (Chiba institute of Technology), Dr. Fujita T (Kyoto University), and Dr. Sasai M (Osaka University) for critical comments, and Suzuki T for help on microscopic analysis. O and Oc cells were kindly gifted from Kato N [44].

Author Contributions

Conceived and designed the experiments: HO M Mi Ma TS. Performed the experiments: HO M Mi. Analyzed the data: HO M Mi. Contributed reagents/materials/analysis tools: HO M Mi. Wrote the paper: HO M Mi TS.

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Toll-IL-1-Receptor-Containing Adaptor Molecule-1: A Signaling Adaptor Linking Innate Immunity to Adaptive Immunity

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Abstract

The innate immune system senses microbial infections using pattern-recognition receptors and signals to activate adaptive immunity. Type I transmembrane protein Toll-like receptors (TLRs) play important roles in antimicrobial immune responses. Upon the recognition of pathogen-associated molecular patterns, TLRs homo- or heterodimerize and recruit distinct adaptor molecules to the intracellular TIR domains. Toll-IL-1-receptor-containing adaptor

molecule-1 (TICAM-1) is a signaling adaptor downstream of TLRs 3 and 4 that recognizes virus-derived double-stranded RNA and lipopolysaccharide, respectively. TLR3 is expressed on the endosomal membrane in myeloid DCs, where TLR3-mediated signaling is initiated. Once TICAM-1 is activated, transcription factors, IRF-3, NF- κ B, and AP-1, are activated, leading to production of IFN- β and proinflammatory cytokines and maturation of dendritic cells, which are capable of activating NK cells and cytotoxic T cells. Hence, TICAM-1 signaling appears to link innate immunity to adaptive immunity. In this review, we summarize the current knowledge on TICAM-1 and discuss its role in virus infection and antitumor immunity.



1. INTRODUCTION

The innate immune system senses microbial infections using pattern-recognition receptors (PRRs), including membrane-bound Toll-like receptors (TLRs), cytosolic RIG-I-like receptors (RLRs), and NOD-like receptors, which rapidly induce an antimicrobial response.^{1,2} PRRs recognize unique microbial components called pathogen-associated molecular patterns (PAMPs) and activate signaling cascades via distinct adaptor proteins, leading to the production of type I IFNs and proinflammatory cytokines in innate immune cells. Moreover, accumulating evidence indicates that PRRs are involved in the recognition of damage-associated molecular patterns (DAMPs), which are closely associated with the development of autoimmune diseases or inflammatory disorders.^{3,4}

TLRs are the first PRRs shown to link innate immunity to adaptive immunity through induction of dendritic cell (DC) maturation and cytokine production.⁵ In humans, 10 members of the TLR family of proteins (TLR1–10) exist. The TLRs are classified into two groups based on subcellular localization. TLR 1, 2, 4, 5, and 6 are expressed on cell surfaces and detect lipids, carbohydrates, or proteins derived from microbes. TLR 3, 7, 8, and 9 are localized in endosomal compartments, where TLRs recognize microbial or host nucleic acids (Table 18.1). TLRs are glycosylated type I transmembrane proteins that are composed of extracellular, transmembrane, and intracellular domains. The extracellular domain contains 19–25 leucine-rich repeat (LRR) modules sandwiched between N- and C-terminal flanking regions and is responsible for ligand recognition.⁶ The cytoplasmic domain contains a linker region composed of 20–40 amino acids and a Toll-IL-1 receptor (TIR) domain which is required for interaction with the adaptor proteins. Upon ligand binding, TLRs form homo- or heterodimers and transmit signals via recruitment of

Table 18.1 TLR recognition of microbial components

TLR	Microbial ligands	Subcellular localization
1/2	Triacyl lipopeptides	Cell surface
2	Peptidoglycan, Porin, Lipoarabinomannan	Cell surface
3	Double-stranded RNA	Cell surface, early endosome
4	Lipopolysaccharide	Cell surface
5	Flagellin	Cell surface
6/2	Diacyl lipopeptides, Lipoteichoic acid	Cell surface
7	Single-stranded RNA	ER, endolysosome
8	Single-stranded RNA	ER, early endosome
9	CpG-DNA	ER, endolysosome
10	Unknown	Cell surface

distinct adaptor proteins to the intracellular TIR domains.⁷ Five TIR-containing adaptor molecules, including MyD88, TICAM-1 (also known as TIR domain-containing adaptor-inducing interferon- β [TRIF]), TICAM-2 (also known as TRIF-related adaptor molecule [TRAM]), Mal (also called TIRAP), and SARM, have been identified.⁸ All TLRs, except for TLR3, use MyD88 as a signaling adaptor and induce NF- κ B-dependent cytokine production. TLR7 and TLR9 also induce IRF-7-dependent IFN- α production via MyD88 in plasmacytoid DCs.⁹ TICAM-1 is a TLR3 adaptor that activates the transcription factors, IRF-3, NF- κ B, and AP-1, leading to the induction of type I IFN (especially IFN- β), cytokine/chemokine production, and DC maturation.^{10,11} TICAM-1 is also engaged in TLR4-mediated MyD88-independent signaling cascades downstream of TICAM-2.^{12,13} TICAM-2 is directly associated with TLR4 and bridges between TLR4 and TICAM-1. Mal/TIRAP is another bridging adaptor downstream of TLR4 and TLR2^{14,15} (Fig. 18.1). The last adaptor to be identified, SARM, is a negative regulator for TLR3–TICAM-1 signaling.¹⁶ Thus, the combinations between TLRs and adaptor proteins determine the properties of PAMP-induced innate immune responses. Among these adaptors, TICAM-1 is unique in inducing a wide range of cellular responses linking innate and adaptive immunity. In this review, we summarize the current knowledge on TICAM-1 function in the innate and adaptive immune systems and discuss the role of TICAM-1 in virus infection and antitumor immunity.

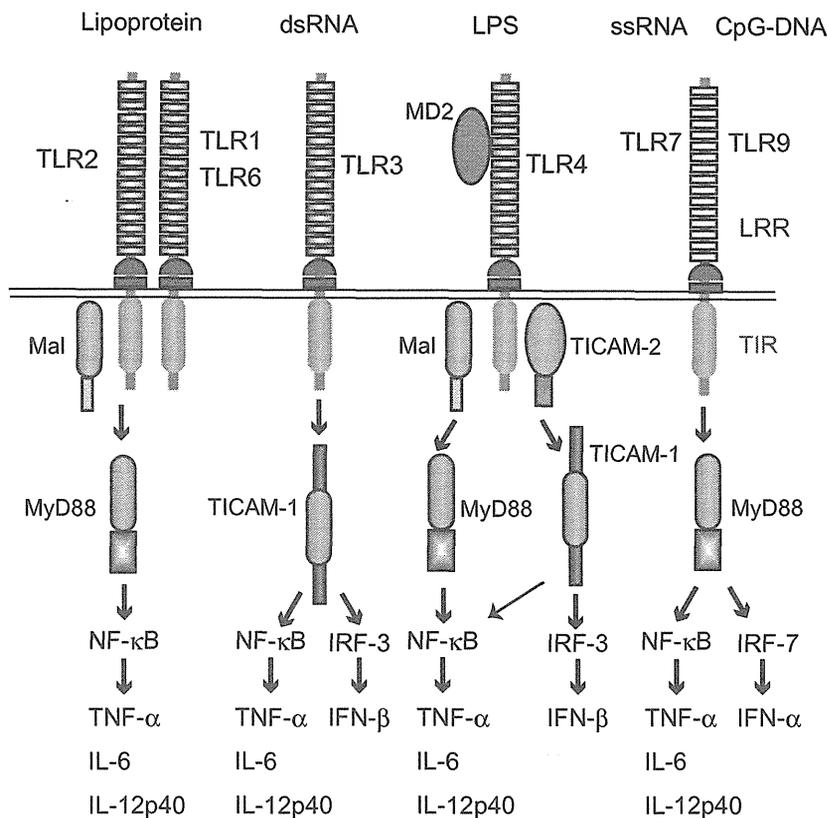


Figure 18.1 TLR transmits signals through distinct adaptor proteins. TLR2/1 and TLR2/6 heterodimers use two adaptor proteins, Mal and MyD88, that activate NF- κ B, leading to production of proinflammatory cytokines, including TNF- α , IL-6, and IL-12p40. TLR3 uses TICAM-1, which activates NF- κ B and IRF-3 and induces proinflammatory cytokines and IFN- β . TLR4 uses four adaptor proteins, Mal, MyD88, TICAM-2, and TICAM-1. TLR7 and TLR9 use MyD88, which activates NF- κ B and also IRF-7 in plasmacytoid DCs and induces IFN- α production.



2. TLR3–TICAM-1 PATHWAY

2.1. Expression and localization of TLR3

TLR3 has been functionally identified as a sensor of virus-derived dsRNA and its synthetic analog, polyriboinosinic:polyribocytidylic acid [poly(I:C)].^{17,18} Upon dsRNA recognition, TLR3 induces IFN- β and proinflammatory cytokine production from host cells via the adaptor protein, TICAM-1. It was subsequently shown that the cytosolic 5'-triphosphated viral RNA and long dsRNA are recognized by the cytosolic RNA helicases, retinoic-acid inducible gene-1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), respectively, which induces IFN- α/β production via the adaptor protein, IPS-1 (also referred to as MAVS, Cardif, and VISA) located on the

mitochondrial outer membrane.^{19–26} More recently, it has been reported that the cytoplasmic RNA helicase complex, DDX1, DDX21, and DHX36, detects dsRNA and induces type I IFNs via TICAM-1, but the precise TICAM-1 activation mechanism is unclear.²⁷

TLR3 is expressed in various tissues and cells. In the human central nervous system, TLR3 is expressed constitutively in neurons, astrocytes, and microglia, suggesting a role in the response to viruses causing encephalopathy.^{28,29} In immune cells, only myeloid DCs and macrophages express TLR3. Monocytes, polymorphonuclear leukocytes, and T, B, and NK cells do not express TLR3.^{30–34} Among DC subsets, TLR3 is highly expressed in the professional antigen-presenting DCs, including human CD141⁺ DCs and mouse splenic CD8 α ⁺ DCs.^{35,36} In contrast, the plasmacytoid DCs, which express TLR7 and TLR9 and secrete large amounts of IFN- α in response to viral ssRNA and imidazoquinoline compounds (TLR7 ligands) or CpG-DNA (TLR9 ligand), do not express TLR3.³⁴ TLR3 is also expressed in fibroblasts and a variety of epithelial cells, including airway, corneal, cervical, biliary, and intestinal cells, which are target sites of virus infection.³⁷ Notably, TLR3 expression is upregulated by type I IFN, and this positive feedback system is important for TLR3-mediated antiviral responses.³⁸

The subcellular localization of TLR3 depends on the type of cell. TLR3 is localized to cell surfaces and endosomes in fibroblasts, macrophages, and some epithelial cell lines.¹⁸ In contrast, myeloid DCs only express TLR3 intracellularly.³⁴ Immunofluorescence analysis has shown that endogenous TLR3 is localized to the early endosome, but not to late endosomes/lysosomes. The ER resident protein, UNC93B1, is physically associated with TLR3 and facilitates intracellular trafficking of TLR3³⁹; however, the linker region between the transmembrane and TIR domains of TLR3 determines the endosomal localization.^{40,41} In any cell type, TLR3 signaling arises in the endosomal compartment, requiring endosomal maturation. Hence, based on endosomal localization and signaling of TLR3, it is generally accepted that the TLR3–TICAM-1 pathway is involved in the detection of extrinsic viral dsRNA, while the RLR–IPS-1 pathway participates in cytoplasmic viral RNA sensing.⁴²

2.2. Recognition of dsRNA

Human TLR3 consists of an extracellular domain containing 23 LRRs and N- and C-terminal flanking regions (LRR-NT and LRR-CT), a transmembrane domain, and an intracellular linker region and TIR domain. It possesses 15 putative carbohydrate-binding motifs in the extracellular

domain. Structural analyses of the human TLR3 ectodomain (ECD) revealed that the LRRs form a large horseshoe-shaped solenoid, one face of which is largely masked by carbohydrate, while the other face is glycosylation-free.^{43,44} A subsequent structural analysis of the mouse TLR3–ECD and 46-bp dsRNA oligonucleotide complex demonstrated that dsRNA interacts with N- and C-terminal binding sites on the glycan-free surface of each mTLR3–ECD, which are located on opposite sides of dsRNA.⁴⁵ Further, the two LRR–CT domains are brought into proximity and form a series of protein–protein interactions which facilitate dimerization of the cytoplasmic TIR domain. Point mutation analyses revealed that the His539 and Asn541 residues in TLR3–LRR20, which are located on the glycan-free lateral face, and the N-terminal-conserved histidine residues, His39 in LRR–NT, His60 in LRR1, and His108 in LRR3, are critical amino acids for dsRNA binding and signaling.^{46–48} Protonation of these imidazole groups under acidic conditions, such as exists in endosomes, appears to generate an ionic interaction between the histidine residues and the negatively charged phosphate backbone of dsRNA. In addition, Asp648, Thr679, and Pro680 in the LRR–CT dimerization site are essential for TLR3 signaling, and this site also influences ligand binding.⁴⁹

Using biochemical techniques, Leonard *et al.*⁵⁰ showed that TLR3–ECD binds to a 40–50 bp length of dsRNA as a dimer, and multiple TLR3–ECD dimers bind to long dsRNA strands. The binding affinities increase with both buffer acidity and dsRNA length. Based on these structural and biochemical analyses of the TLR3–dsRNA complex, it has been proposed that the 40–50 bp dsRNA is the minimum signaling unit with two TLR3 molecules. An alternative model for TLR3 dimer formation has also been proposed, in which shorter RNA duplexes of between 21 and 30 bp can form less stable complexes with two TLR3 molecules.⁴⁷ Recently, Jelinek *et al.*⁵¹ reported that dsRNAs, >90 bp in length, triggered TLR3 oligomerization and efficiently induced IFN- β production and T cell activation in conventional murine DCs, while 60-bp dsRNA did not. Because the >90-bp length of dsRNA, but not the 40- to 50-bp length of dsRNA, forms a stable complex with TLR3 at the pH within early endosomes (\sim 6.0–6.5),⁵⁰ oligomerization of TLR3 at the early endosomes must be required for IFN- β production and efficient DC maturation.

2.3. Mechanism of dsRNA uptake

Because TLR3 is a type I transmembrane protein located on the endosomal membrane, TLR3 is intrinsically involved in the recognition of extracellular

viral dsRNA; however, the molecular mechanism by which extracellular dsRNA is delivered to TLR3-positive organelles remains unknown. Itoh *et al.*⁵² demonstrated that poly(I:C) is internalized via clathrin-dependent endocytosis. In addition, they reported that poly(I:C) shares its uptake receptor with B- and C-type oligodeoxynucleotides (ODNs). Subsequently, Watanabe *et al.*⁵³ identified the cytoplasmic lipid raft protein, Raftlin, that is essential for poly(I:C) cellular uptake and TLR3-mediated IFN- β production in human myeloid DCs and epithelial cells. Raftlin induces the internalization of poly(I:C) by cooperating with the clathrin-AP-2 complex. Confocal microscopic analyses showed that upon poly(I:C) stimulation, Raftlin is translocated from the cytoplasm to the plasma membrane, where it colocalizes with poly(I:C), and thereafter moves to TLR3-positive endosomes with internalized poly(I:C). Raftlin is physically associated with clathrin in response to poly(I:C) stimulation in the cell membrane, and dissociates from clathrin before reaching TLR3-positive endosomes. Interestingly, Raftlin participates in the cell entry of B- and C-type ODNs, but not transferrin.⁵³ Hence, Raftlin appears to modulate cargo sorting and delivery by the clathrin-AP-2 complex.

Several studies have shown that CD14 and the scavenger receptor class A act as an uptake receptor for poly(I:C) in mouse macrophages and human bronchial epithelial cells, respectively.^{54,55} However, these receptors do not appear to participate in poly(I:C) cellular uptake in human DCs and epithelial cells.⁵⁶ Identification of the poly(I:C)/ODN uptake receptor is important for a full understanding of the mechanism of uptake for extracellular nucleic acids.



3. TLR4-TICAM-1 PATHWAY

TLR4 recognizes LPS cooperatively with MD-2.^{5,57} Upon LPS binding, TLR4 undergoes dimerization and associates with a bridging adaptor, Mal/TIRAP, that contains a phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] binding domain.⁵⁸ MyD88 translocates to the cell surface and colocalizes with Mal/TIRAP and TLR4 in PtdIns(4,5)P₂-rich regions, thus allowing for activation of the NF- κ B pathway and leading to production of proinflammatory cytokines. TLR4 then moves into the endosome in a dynamin- and clathrin-dependent manner and induces IRF-3 and NF- κ B activation, leading to the production of IFN- β .⁵⁹ TICAM-2 and TICAM-1 participate in this MyD88-independent signaling pathway.⁶⁰

TICAM-2 was discovered via homology search using the TICAM-1-TIR domain.¹² Human TICAM-2 mRNA is expressed in many tissues and cells, including DCs, macrophages, and natural killer cells, and various B and T cell lines. Human TICAM-2 consists of 235 amino acids with a short N-terminal Ser/Thr-rich domain, a TIR domain, and a C-terminal 20-amino acid stretch (Fig. 18.2A). The TIR motif of TICAM-2 has low similarity to that of Mal and MyD88. TICAM-2 contains a myristoylation motif at the N-terminus, which is essential for localization and function.

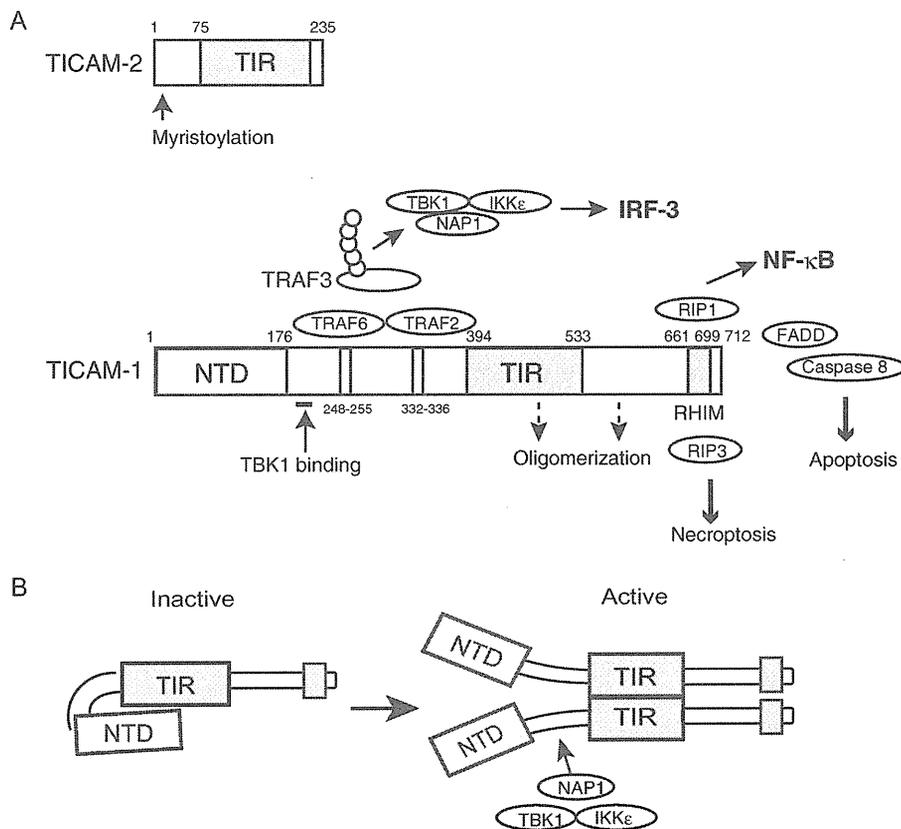


Figure 18.2 Schematic representation of protein domains and motifs in human TICAM-1 and TICAM-2 and the signaling cascades. (A) TICAM-1 and TICAM-2 have a TIR domain. TICAM-2 is myristoylated at the N-terminus. TICAM-1 has an N-terminal domain (NTD) that regulates TICAM-1 activation. TBK1, TRAF2, and TRAF6-binding sites are located between the NTD and TIR domain. TRAF3 and NAP1 participate in the recruitment and activation of IRF-3 kinase, TBK1, and IKK ϵ . The RHIM domain is located at the C-terminus, which is essential for NF- κ B activation and induction of apoptosis or necroptosis. (B) Model of TICAM-1 activation. In resting cells, TICAM-1 is inactivated by intramolecular interaction between the NTD and TIR domain (left panel). Upon stimulation of TLR3/4, or TICAM-1 overexpression, TICAM-1 oligomerizes through the TIR domain and the C-terminal region, which breaks the intramolecular association and induces a conformational change that allows access of downstream signaling molecules to their binding sites (right panel). See text for a detailed explanation.

Kagan *et al.*⁵⁹ showed that wild-type TICAM-2 is localized to the plasma membrane and early endosome, while a myristation-deficient TICAM-2 mutant is uniformly distributed throughout the cell and fails to mediate signaling. Functional analyses using various TICAM-2 mutants revealed that endosomal but not cell surface localization of TICAM-2 is sufficient to induce TLR4-mediated TICAM-2–TICAM-1 signaling. Upon LPS stimulation, TICAM-2 associates with internalized TLR4 at the early endosome and recruits TICAM-1. Pro714 in the TLR4-TIR domain is critical for interaction with TICAM-2-TIR.¹²

In addition to LPS stimulation, the forced expression of TICAM-2 also activates NF- κ B and IRF-3 via endogenous TICAM-1. The TICAM-2-TIR mutant C117H, which has TLR4 binding ability, fails to homodimerize and does not interact with TICAM-1, suggesting that homodimerization of TICAM-2 is critical for TICAM-1 recruitment.¹²



4. TICAM-1 SIGNALING

4.1. Structure of TICAM-1

Human TICAM-1 is composed of 712 amino acids, which consists of an N-terminal region, a TIR domain, and a C-terminal region (Fig. 18.2A). The TIR domain of TICAM-1 is essential for binding to the TIR domain of TLR3 and to the TLR4 adaptor TICAM-2. Pro434 in the TIR domain is essential for homo-oligomerization of TICAM-1, but not for association with TLR3 or TICAM-2.⁶¹ The N-terminal region is crucial for TICAM-1-mediated IRF-3 activation, which contains the N-terminus regulatory domain (NTD) and the TBK1, TRAF6 and TRAF2 binding sites.^{62–64} The C-terminal region contains the RIP homotypic-interacting motif (RHIM) domain and is involved in NF- κ B activation and apoptosis.^{65–67} TICAM-1 is expressed at a low level in most tissues and cells, and is diffusely localized in the cytoplasm of resting cells.⁶⁸ When endosomal TLR3 is activated by dsRNA, TICAM-1 transiently colocalizes with TLR3, then dissociates from the receptor, and forms speckled structures that colocalize with downstream signaling molecules. Thus, TICAM-1 alters the distribution profile in the cytosol in response to dsRNA.

4.2. TICAM-1 oligomerization and signaling

TICAM-1 activates the transcription factors, IRF-3, NF- κ B, and AP-1, leading to the induction of type I IFN, cytokine/chemokine production,

and DC maturation, which then enables the activation of NK cells and CTLs. In contrast to other TLR adaptors, the forced expression of TICAM-1 leads to potent induction of IFN- β promoter activation. Homo-oligomerization of TICAM-1 is critical for TICAM-1-mediated activation of NF- κ B and IRF-3.⁶¹ In a yeast two-hybrid system, TICAM-1-TIR mutant P434H failed to interact with the TIR domain of TICAM-1, but bound tightly to the TIR of TLR3 or TICAM-2, suggesting that Pro434 is critical for TICAM-1 dimerization. In addition, the C-terminal region of TICAM-1, with the exception of the RHIM, mediates TICAM-1 oligomerization. Mutation of Pro434 or deletion of the C-terminal region reduces TICAM-1-mediated NF- κ B and IRF-3 activation. Thus, Pro434 and the C-terminal region are required for TICAM-1 oligomerization. Once TICAM-1 is oligomerized, the serine-threonine kinases, TANK-binding kinase 1 (TBK1; also called NAK or T2K) and I κ B kinase-related kinase ϵ (IKK- ϵ ; also called IKK- ι), are activated and phosphorylate IRF-3.^{69,70} The phosphorylated IRF-3 then translocates into the nuclei, leading to IRF-3-dependent gene expression. The ubiquitin ligase of the TRAF family members, TRAF2, TRAF3, and TRAF6, are downstream signaling molecules of TICAM-1 (Fig. 18.2A). TRAF2 and TRAF6 directly bind to the N-terminal region of TICAM-1 and mediate Lys63-linked TICAM-1 polyubiquitination, which facilitates TICAM-1-mediated IFN- β promoter activation.⁶³ Lys63-linked autoubiquitination of TRAF3 is also required for IRF-3 activation.^{71,72} Further, NF- κ B activating kinase (NAK)-associated protein 1 (NAP1) participates in the recruitment of IRF-3 kinases to the N-terminal region of TICAM-1.⁷³ Although TRAF3 and NAP1 associate with oligomerized TICAM-1 and serve as a critical link between TICAM-1 and downstream IRF-3 kinases, there is no evidence that they bind directly to TICAM-1. Interestingly, Tatematsu *et al.*⁶⁴ showed that direct binding of TBK1 to TICAM-1 is necessary for IRF-3 activation. The Leu194 residue in the N-terminal region is critical for TBK1 binding to TICAM-1 (Fig. 18.2A). The L194A mutant of TICAM-1 failed to recruit TBK1, resulting in disabling IRF-3 phosphorylation, although TRAF3 and NAP1 were recruited. In addition, the Ser189, Arg195, and Ser196 residues are involved in TBK1-TICAM-1 binding.

The N-terminal 176 amino acids of TICAM-1 (NTD) form a protease-resistant structural domain. Because the crucial amino acids for TRAF2, TRAF6, and TBK1 binding reside between the NTD and TIR domain, naive TICAM-1 may have a closed conformation that covers these binding sites (Fig. 18.2B). Indeed, protein-protein interaction analysis has revealed

that the NTD interacts with the N-terminus of TICAM-1-TIR.⁶⁴ Thus, the NTD folds into the TIR domain structure to maintain the naive conformation of TICAM-1. Upon stimulation of TLR3 or TLR4, TICAM-1 oligomerizes through the TIR domain and the C-terminal region, possibly breaking the intramolecular association and inducing a conformational change that allows TBK1 access to TICAM-1 (Fig. 18.2B).

Whereas the N-terminal region is crucial for TICAM-1-mediated IRF-3 activation, the C-terminal region of TICAM-1 is involved in NF- κ B activation and apoptosis. Receptor-interacting protein 1 (RIP1), a kinase containing a death domain, associates with TICAM-1 via the RHIM domain in the C-terminal region and acts as an NF- κ B inducer and apoptosis mediator in TICAM-1-mediated signaling.^{65–67} TRAF6 has also been implicated in NF- κ B activation by TICAM-1 in a cell type-dependent manner.^{62,74} Notably, the TICAM-1 mutant lacking the RIP1 binding motif failed to recruit RIP1 and TBK1. Thus, full activation and formation of TICAM-1 signalosomes require oligomerization induced at two different sites and RIP1 binding.

TLR3–TICAM-1-mediated type I IFN production is negatively regulated by deubiquitinating enzyme A (DUBA) (Fig. 18.3). DUBA selectively cleaves the Lys63-linked polyubiquitin chains on TRAF3, resulting in its dissociation from the downstream signaling molecules.⁷⁵ In addition, the ubiquitin-modifying enzyme A20 inhibits TICAM-1-mediated NF- κ B activation by deubiquitinating TRAF6.⁷⁶ However, the precise mechanisms by which TRAF3 and TRAF6 are ubiquitinated and interact with downstream signaling molecules are unknown.

4.3. Induction of apoptosis/necroptosis

Overexpressed TICAM-1 potently induces apoptosis. TICAM-1-mediated cell death is inhibited by a dominant-negative form of Fas-associated death domain protein (FADD), a caspase inactive mutant of caspase-8, or the caspase inhibitor CrmA, indicating that TICAM-1 induces apoptosis via the FADD–caspase-8 axis.⁶⁶ This signaling event has been shown to be tightly regulated by the cellular FLICE-like inhibitory protein short form (cFLIPs).⁷⁷ cFLIPs heterodimerizes with caspase-8 to suppress the self-activation of caspase-8. Mutational analyses have revealed that the RHIM located in the C-terminus of TICAM-1 is essential for TICAM-1-induced apoptosis.⁶⁶ TICAM-1 physically interacts with RHIM-containing proteins, RIP1 and RIP3, of which RIP1, but not RIP3, contains the death

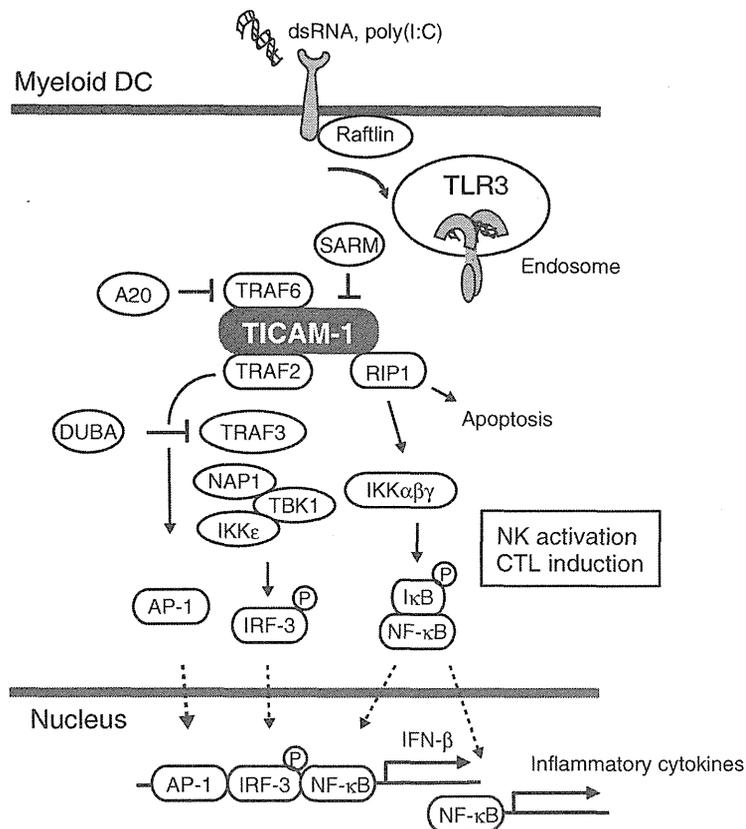


Figure 18.3 TLR3–TICAM-1 signaling pathway. In myeloid DCs, TLR3 is expressed in the early endosome and recognizes viral dsRNA and its synthetic analog, poly(I:C), which are internalized via raftlin- and clathrin-dependent endocytosis. Once TLR3 is dimerized by dsRNA, TLR3 recruits the adaptor protein, TICAM-1. After a transient association between TLR3 and TICAM-1 through the TIR domain, TICAM-1 dissociates from TLR3 to form a speckle-like signalosome with downstream signaling molecules, including RIP1, TRAF2/3/6, NAP1, and TBK1, where TICAM-1-mediated signaling is initiated. RIP1 associates with TICAM-1 via the PHIM domain and acts as a NF- κ B activator and apoptosis mediator in TICAM-1-mediated signaling. Phosphorylated IRF-3 translocates into the nucleus, and together with NF- κ B and AP-1, induces IFN- β gene transcription. TICAM-1-mediated AP-1 activation pathway is unclear. In addition to IFN- β and inflammatory cytokine production, the TICAM-1 signal induces DC maturation capable of activating NK cells and CTLs. SARM, A20, and DUBA inhibit TICAM-1 signaling via targeting distinct molecules.

domain. Overexpression of TICAM-1 with mutant RHIM cannot recruit RIP1 and failed to induce apoptosis, indicating that TICAM-1 induces RIP1–FADD–caspase 8-dependent apoptosis (Fig. 18.2A). The ability of TICAM-1 to induce apoptosis appears to be segregated from those of the activation of IRF-3 and NF- κ B.

Several very recent studies have demonstrated that RIP1 and RIP3 mediate cell death, which is referred to as programmed necrosis or

necroptosis.^{78–80} In RIP1/3-dependent necrosis, ligating TLR3 or TLR4 activates RIP1 via TICAM-1, which in turn binds to RIP3, thus triggering cell death.⁸¹ These pathways are inhibited by caspase-8, and therefore this type of necrosis appears under some conditions of caspase-8 inhibition. Feoktistova *et al.*⁷⁷ reported that the loss of cellular inhibitor of apoptosis proteins (cIAPs) promotes TLR3-induced RIP1–RIP3-mediated necroptosis. Intriguingly, they showed that in the absence of cIAPs, cFLIPs was able to protect from the RIP1-induced apoptosis, but promoted necroptosis, that is caused by the lack of caspase activity. TLR3 is expressed in many tumor cells at a high level, including melanoma, hepatocellular carcinoma, and breast cancer.^{82–84} The proapoptotic activity of TLR3/TICAM-1/RIP1/caspase-8 in cancer cells is under the control of inhibitor of apoptosis proteins.

4.4. Induction of type I and III IFNs and proinflammatory cytokines

Upon TLR3 stimulation, TICAM-1 induces gene expression of IFN- β in fibroblasts, epithelial cells, and myeloid DCs, which generate antiviral states in both stimulated and surrounding unstimulated cells by induction of IFN-stimulated genes through IFN- α/β receptor.⁸⁵ TICAM-1 activation also induces IL-12p70 production from human, but not mouse myeloid DCs. IL-12p70 is an important cytokine that directs naïve Th0 cells to differentiate into Th1 cells in conjunction with antigen stimulation. Further, mouse CD8 α^+ DCs and human CD141 $^+$ DCs produce type III IFN (IFN- λ) in response to poly(I:C), which depends on TLR3–TICAM-1.⁸⁶

Interestingly, when poly(I:C) is added to cells or injected into mice, both endosomal TLR3 and cytoplasmic MDA5 recognize poly(I:C) and induce gene expression via TICAM-1 and IPS-1, respectively.^{87,88} After *in vivo* poly(I:C) injection or *in vitro* bone marrow-derived DC (BMDC) stimulation, production of IFN- α is not impaired in TICAM-1 $^{-/-}$ mice compared with wild-type mice, whereas IL-12p40 production is completely dependent on TICAM-1; in contrast, IFN- β and IL-6 productions are partially diminished in TICAM-1 $^{-/-}$ mice.

4.5. Induction of adaptive immunity

TICAM-1 signaling is important for DC-mediated activation of NK cells and CTLs (Fig. 18.4). TICAM-1-induced IFN- β and IL-12p70 directly activate NK cells. In addition, the TLR3–TICAM-1 pathway in myeloid DCs facilitates the DC–NK cell interaction following NK cell activation.⁸⁹

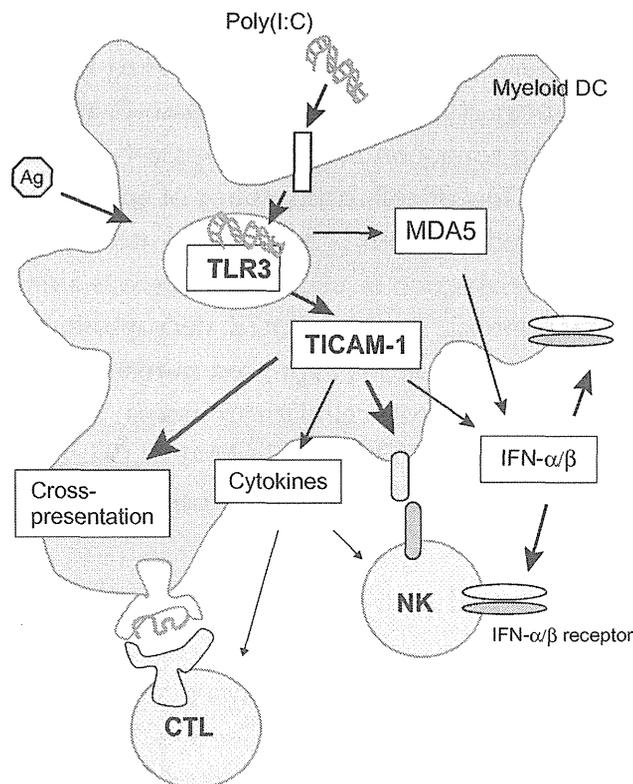


Figure 18.4 Double-stranded RNA-induced TLR3–TICAM-1-mediated cellular responses in myeloid DCs. Myeloid DCs take up extracellular poly(I:C) or apoptotic bodies containing dsRNA from virus-infected cells and induce IFN- α/β and cytokine production, NK cell activation, and CTL induction via the TLR3–TICAM-1 pathway. Extracellular poly(I:C) is further delivered to the cytoplasm and activates MDA5, leading to production of IFN- α/β . IFN- α/β participates in NK cell activation and promotion of cross-priming resulting in CTL induction, which largely depends on the experimental conditions.

Ebihara *et al.*⁹⁰ reported that TICAM-1–IRF-3-dependent expression of a novel molecule, namely IRF-3-dependent NK activation molecule (INAM), in myeloid DCs is required for NK cell activation.

Myeloid DCs are the most effective professional antigen-presenting cells, possessing several antigens processing, and transporting machineries.^{91,92} One of the most notable features of myeloid DCs is the cross-presentation of exogenous antigens to CD8⁺ T cells. This pathway is important for effective host CTL induction against viruses that do not directly infect DCs and tumor cells. Among the myeloid DC subsets, the splenic CD8 α ⁺ DC subset in mice and the CD141(BDCA3)⁺DNGR-1(CLEC9A)⁺ DC subset in humans highly express TLR3 and display a superior capacity for cross-presenting apoptotic and necrotic cell antigens after TLR3 stimulation.^{36,37} Using TLR3-deficient mice, Schultz *et al.*⁹³ clearly showed that TLR3 plays an important role in cross-priming. In many cases, virus-infected cells

produce IFN- α/β that activates DCs to promote CD8⁺ T cell cross-priming.⁹⁴ Thus, both TLR3- and IFN- α/β -mediated signalings are likely implicated in licensing DCs for the cross-priming of CD8⁺ T cells. However, the molecules responsible for TICAM-1-mediated cross-priming have not been identified.



5. TICAM-1 AND HOST DEFENSE

5.1. Role of TLR3–TICAM-1 in virus infection

Double-stranded RNA is generated in the cytoplasm during replication of positive-strand RNA and DNA viruses.⁹⁵ There is accumulating evidence that the TLR3–TICAM-1 signaling is involved in protection against virus infection. Two independent studies have demonstrated that the TLR3–TICAM-1 pathway is essential for protection against poliovirus infection, a virus belonging to the *Picornaviridae* family.^{96,97} Poliovirus receptor (PVR)-transgenic and TICAM-1- or TLR3-deficient mice are more susceptible to PV than PVR-transgenic mice. TLR3-dependent type I IFN production by splenic CD11c⁺ DCs and macrophages is essential for the protection of PVR-transgenic mice against PV infection. Moreover, Negishi *et al.*⁹⁸ showed that TLR3^{-/-} mice are more vulnerable to coxsackievirus group B serotype 3 (a virus belonging to the *Picornaviridae* family) than wild-type mice, in terms of higher mortality and acute myocarditis. In addition to these mouse studies, genetic studies in patients with herpes simplex encephalitis demonstrated that the TLR3–TICAM-1 pathway is involved in protection against herpes simplex virus-1 encephalitis in children.^{99–101} TLR3 is expressed in the central nervous system, where it might be required to control HSV-1.

In contrast, a detrimental effect of TLR3 signaling on the outcome of viral infection has been reported in several animal models. Wang *et al.*¹⁰² showed that TLR3^{-/-} mice have impaired cytokine production and enhanced viral loads in the periphery after West Nile virus (a positive-stranded RNA virus) infection, whereas in the brain, the viral load, inflammatory responses, and neuropathology were reduced compared with wild-type mice. TLR3-mediated peripheral inflammatory cytokine production is critical for disruption of the blood–brain barrier, which facilitates viral entry into the brain, thus causing lethal encephalitis. In other RNA viral infections such as respiratory syncytial virus, influenza A virus, and phlebovirus (all negative-stranded RNA viruses), TLR3-dependent inflammatory cytokine, and chemokine production affect virus-induced pathology

and host survival.^{103–105} Thus, TLR3 plays different roles in virus infections, which depends on the viral genome structure, the TLR3-expressing cell type that encounters viral RNA, and the properties of host antiviral effector functions.

5.2. Antiviral cellular immunity induced by the TLR3–TICAM-1 pathway

CTLs and NK cells are principal effector cells in antiviral immunity. The contribution of TLR3 to antiviral responses has been shown in MCMV infection,¹⁰⁶ during which virus clearance is partly dependent on NK cell activation. TLR3^{-/-} mice are hypersusceptible to MCMV infection. Cytokine production and NK and NKT cell activations are impaired in TLR3^{-/-} mice compared with wild-type mice. Recently, it has been shown that TLR3- and TICAM-1-mediated signaling in the nonhematopoietic compartment contributes to protection from intestinal epithelial rotavirus infection in adult mice.¹⁰⁷ In rotavirus-infected TLR3^{-/-} mice, the expressions of IFN- λ and chemokines are reduced, and the recruitment of CD8⁺ T and NK cells to intestinal tissue is impaired, which might contribute to enhanced virus replication. In humans, Ebihara *et al.*¹⁰⁸ demonstrated the role of TLR3, expressed in myeloid DCs, in the immune response to HCV infection. The phagocytosis of HCV-infected apoptotic cells that contain HCV-derived dsRNA and their interaction with the TLR3 pathway in myeloid DCs, plays a critical role in DC maturation and activation of T and NK cells. In addition, Jongbloed *et al.*³⁵ reported that CD141⁺ DCs are able to cross-present viral antigens from human cytomegalovirus-infected necrotic fibroblasts. Physiologically, TLR3 in a DC subset specialized for antigen presentation encounters viral dsRNA in the endosome by uptake of apoptotic or necrotic virus-infected cells and signals for cross-presentation of viral antigens.

5.3. TLR3–TICAM-1 pathway in antitumor immunity

Selective expression of TLR3 in professional antigen-presenting DCs is an advantage when using TLR3 ligands as an antitumor vaccine adjuvant (Fig. 18.4). Azuma *et al.*¹⁰⁹ recently demonstrated that the TICAM-1 pathway in mouse CD8 α ⁺ DCs is crucial for cross-priming for antitumor CTLs induced by tumor-associated antigen and poly(I:C). When tumor cell lysate and poly(I:C) are subcutaneously injected in tumor-bearing mice, tumor growth retardation is observed in wild-type mice, and to a lesser extent IPS-1^{-/-} mice, but not TICAM-1^{-/-} mice. IRF3 and IRF7 are essential,