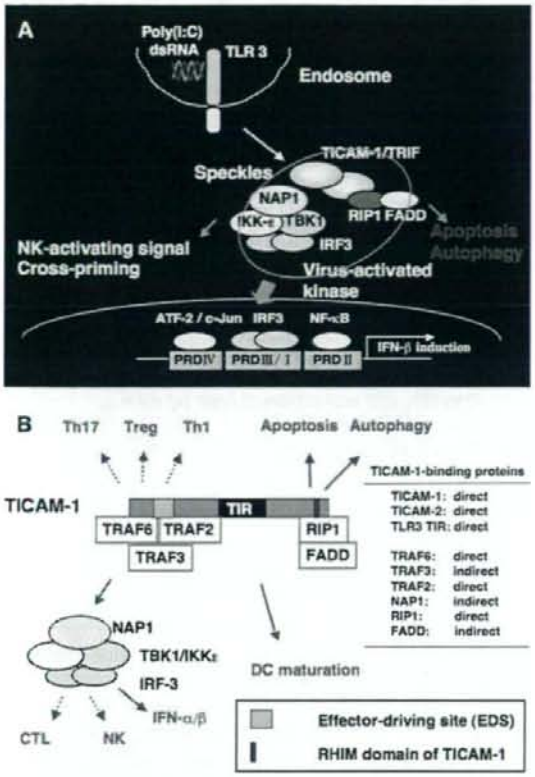

Fig. 2 An outline of the TICAM-1 pathway. **a** In human cells, TICAM-1 once detached from TLR3 serves as a signaling platform to induce apoptosis, autophagy, NK activation and cross-priming. TICAM-1 undergoes some modiWcation secondary to complex formation with TLR3, forms multimer, and dissociated from TLR3 with unknown mechanism. The pathways for NK activation, CTL induction and autophagy are only partially identiWed, although the pathway for apoptosis is getting clariWed. Although epithelial cells in bronchi, bile-duct and intestine express TLR3 on their surface membranes, it is undetermined whether surface-expressed TLR3 retains the cellular responses. **b** The N-terminal 'EVector-driving site (EDS)' recruits appropriate signal-transmitting molecules and matures mDCs leading to induction of eVector cells, including NK and CTL. The C-terminal RHIM domain participates in signal transmission for apoptosis and autophagy. TICAM-1-binding proteins, either direct or indirect, are summarized in the inset table

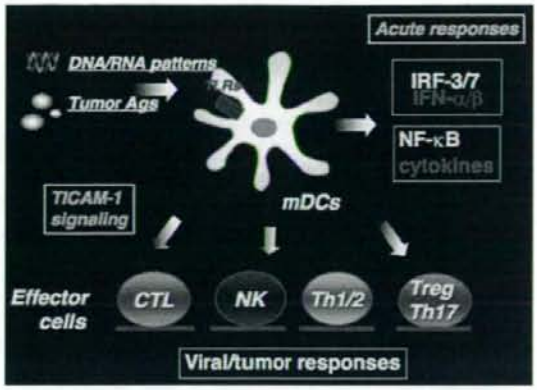

Fig. 3 Various eVectors driven by the TICAM-1 pathway in mDCs. The eVectors can be induced through the TICAM-1 pathway in mDCs are delineated in this Wgure. In an early phase of infection, cytokines and IFNs are released in response to microbial patterns. Later, the cellular eVectors are induced secondary to activation of the TICAM-1 pathway in mDCs

when they are targeted to the endosome [6]. Synthetic or viral replication-induced RNA products with the stem or stem-loop structures possess mild TLR3-agonistic activity and have no toxic eVect on mice. These modiWed RNA duplex signatures are potential TLR3 stimulants.

Although the natural ligands of TLR3 remain unknown, TLR3 recognizes RNA duplex. To date, it has been shown that polyI:C and the duplex signatures of RNA from many viruses and other synthetic RNAs can be recognized by TLR3. DOTAP and other lipofection agents can deliver RNA to the endosome where TLR3 is localized in mDCs [6]. TLR3 links to the adaptor TICAM-1 to induce IFN-• signaling. Whether or not TLR3 links to MyD88 in addition to TICAM-1 in mDCs remains unknown. However, based on the results from knock-out (KO) mice analyses, the contribution of the MyD88 pathway to the functioning of mDCs is minimal, if any [31].

TICAM-1 signaling

TICAM-1 is the largest of the four TLR adaptors identiWed so far [32]. It serves as a platform for the assembly of the TRAF family [33, 34] and TANK family [18, 19]. The N-terminal region of TICAM-1 [named EVector-driving site (EDS)] participates in the molecular recruitment (Fig. 2b). In contrast, RIP1 [35] and FADD [36] are recruited to its C-terminal region (Fig. 2b). A variety of cellular outputs were then developed [34, 37]. IFN-•/•, proinXammatory cytokines, ROS and K+ are induced in mDCs. Autophagy and apoptosis are evoked in cells other than mDCs. TICAM-1 modiWcation and translocation lead to the formation of TICAM-1 homo-multimers in mDCs, which activate signal pathways leading to induction of cellular eVectors, CTL, NK and CD4+ T cells [15]. The IFN-•/•-inducing pathway of TICAM-1 has been well characterized. Interferon regulatory factor (IRF)-3 and -7 are activated by virus-activated kinase (VAK) [38]. A similar pathway induces IL-1•, IL-6, TNF-• and IL-12p40 [38]. However, the pathways by which ROS are induced remains unknown. Recent reports suggest that LPS, a ligand that activates the TLR4-TICAM-1 pathway [39], induces the activation of

12 3

the inXammasome which may interfere with autophagy. This leads to incremental production of IL-1• as well as ROS [40]. Thus, entire pathways led by TICAM-1 remain to be characterized but the pathways appear to coordinately diverge to induce diVerent eVectors.

Two PRRs link the TICAM-1 adaptor in humans and mice. TLR3 directly couples with TICAM-1 [41, 42], whereas TLR4 recruits the TICAM-2 (TRAM)-TICAM-1 complex in human and mouse cells [39]. Once dsRNA is provided exogenously, it is taken up into the endosome where TLR3 is expressed [43]. When TLR3 is stimulated, TICAM-1 is recruited to the cytoplasmic TIR domain of TLRs and then dissociated from the receptor, leading to multimer formation [43, 44]. Multimeric TICAM-1 is capable of assembling TRAF family proteins (particularly TRAF2, 6 and 3) in the N-terminal region of TICAM-1 [33]. This ubiquitin E3 ligase complex binds VAK, consisting of NAP1 (or other TANK family proteins), IKK• and/or TBK1. VAK in turn activates IRF-3 and IRF-7 in the cytoplasm [38]. The phosphorylated IRFs translocate to the nucleus to activate the IFN-•/• promoters. The MAPK pathway may be activated through the N-terminal region of TICAM-1. On the other hand, the C-terminal portion of TICAM-1 recruits RIP1, which leads to the activation of IKK•/• and NK-•B [35]. These pathways sustain the production of inXammatory cytokines and type I IFNs. Although the TICAM-1 protein is maintained at low levels in normal cells, the mechanism by which this protein is regulated remains unknown.

In contrast, MAVS, which is the adaptor molecule of RIG-I/MDA5 for signaling the presence of cytoplasmic dsRNA, also binds TRAF (3 and 6), TRADD and RIP1 in the outer mitochondrial membrane to activate VAK [45]. If this protein is cleaved at the C-terminus by the NS3/4A protease of HCV, it loses the ability to transduce signaling to VAK [46]. It also inactivated by proteolytic cleavage by caspase 1 [47].

The TICAM-1 pathway in cancer cells

Tumor cells induce autophagy via the TICAM-1 pathway [48]. Poly I:C is a compound that induces autophagy in tumor cells, and this reaction augments the activation of caspase 1 of the inXammasome that produces robust amounts of active IL-1•, IL-18 and IL-33 [49]. TICAM-1 KO cells lose the ability to undergo poly I:C-mediated inXammasome activation. This autophagy-augmenting activity is TICAM-1-dependent, and has been mapped to the N-terminal region of EDS.

Breast cancer cells undergo apoptosis upon treatment with poly I:C [50]. Intestinal epithelial cells of mice are injured upon intraperitoneal administration of poly I:C [51]. Previous studies have shown that TICAM-1-overexpressing cells induce apoptosis through a RIP/FADD/caspase 8-dependent pathway [52]. PKR may be additionally involved in dsRNA-derived apoptosis [53]. TLR3 as well as PKC-alpha plays a part in poly(I:C)-mediated tumor cell apoptosis [54]. In other reports, cell damage and apoptosis by poly I:C were not merely due to the TICAM-1 pathway, but were a consequence of the output secondary to other dsRNA-sensing pathways [22, 52–54].

Some tumor cell lines induce IL-6, IL-12p40, IL-1•, TNF-• and IL-8 in response to poly I:C. Of these, IL-12p40 induction is largely dependent on TICAM-1 [55]. Other cytokines partly depend on TICAM-1 and the MAVS pathway.

CTL and NK cell activation driven by mDCs

CTL is induced by TICAM-1

CTLs proliferate in response to Ags presented on MHC class I molecules in mDCs. Endogenous Ags, including proteins of viral origin, are presented on MHC class I molecules to induce MHC-restricted CTL in virus replication. Since dsRNA is produced along with Ag presentation in virus-infected mDCs, viral Ags are eYciently presented in a TAP-dependent manner under these circumstances, and pattern molecules, which are dsRNA molecules in this case, simultaneously stimulate mDCs. However, mDCs are not always infected with viruses and even when they are non-infected, they can present viral Ag in a TAP-independent manner [56]. In other word, when Ags and dsRNA are extrinsically taken up into mDCs, the cross-priming mechanism enables mDCs to present Ags on MHC class I molecules [56, 57]. Cross-priming is enhanced by the TICAM-1 pathway in mDCs (Fig. 2a), which eYciently induce Ag-speciWc CTL [58]. It is expected that similar dsRNA-mediated cross-priming occurs in mDCs that phagocytose TAA instead of viral Ags [3]. Activation of the pathway that induces CTL against TAAs may occur in mDCs and this may facilitate the regression of MHC-high tumors.

Based on increasing evidence obtained by deletion mutagenesis experiments, the N-terminal region of TICAM-1 is involved in the initiation of cross-priming in mDCs. The region contains the site required for TRAF-binding and VAK activation, and probably overlaps with EDS (Fig. 2b). However, induction of cross-priming is independent of IRF-3/7. Thus, occurrence of this event relies on the mechanism involving the molecules for VAK activation but is not dependent on the transcription factors IRF-3/7 [59].

NK cells are induced by TICAM-1

NK cell activation is reciprocally induced by dsRNA-stimulated mDCs (Fig. 4). The mDC-activated NK cells

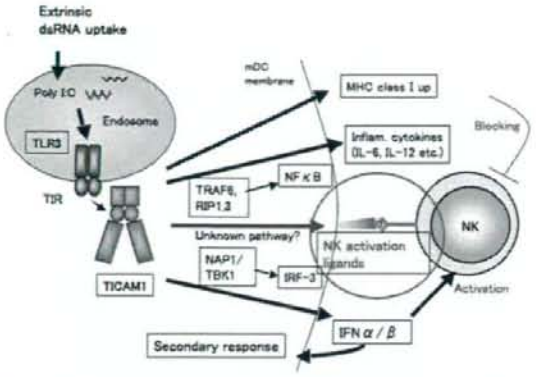

Fig. 4 Possible NK-inducing pathways against cancer. NK activation is an example of mDC output. For full activation, NK cells have to be supported by dendritic cells (myeloid DCs in this Wgure) that recognize pathogen-associated molecular patterns (PAMPs). In general, NK cells and dendritic cells are reciprocally activated by soluble signals and cell/cell contact. Since the tropism of the pathogen varies, the main NK activating players are determined by which sensor cells are attacked by the pathogens and induce innate signals for NK activation

eVectively damage MHC-low tumor cells. The events involved in mDC-mediated NK activation upon stimulation with dsRNA remains unknown. TICAM-1 KO mDCs fail to activate NK cells and TICAM-1 KO NK cells fully restore IFN-• induction and cytotoxic activity against NK target cells [60], suggesting that mDC TICAM-1 is essential for NK driving. Transwell experiments have revealed that cell–cell contact between mDCs and NK cells rather than with mDC-liberated mediators is crucial for mDC–NK activation [60]. The molecule responsible for NK activation must be expressed on the surface of mDCs in response to dsRNA stimuli and foster mDC–NK interaction (Fig. 4). Genechip analyses using TICAM-1 KO versus wild-type mDCs stimulated with polyI:C, have permitted the identiWcation of several molecules as TICAM-1-dependent NK activation enhancers. NK activation followed by mDC maturation has a strong antitumor eVect against MHC-low tumors. The TICAM-1 region required for NK driving in mDCs is the N-terminal region that includes the EDS of TICAM-1 (Fig. 2b). Induction of IRF-3, not IRF-7, is essential for this mDC-NK reciprocal activation [59].

Induction of Th, Treg and Th17 cells by mDCs

CD4+ Th cells

CD4+ Th cells play a pivotal role in skewing the immune responses against cancer. Th1 eVector cells are critical for the maintenance of memory CD8+ T cells [61–63], while Th2 cells help B cells to produce various classes of immunoglobulins (Ig) [64, 65]. It is not completely clear as to how memory T cells are regulated by CD4+ T cells, but the importance of CD4+ T cells in the generation and expansion of CD8+ memory T cells has been reported [66]. Earlier data on CD4+ T cell functions should be interpreted cautiously since in those studies, the CD4+ Th populations frequently contained CD4+ regulatory T (Treg) and Th17 cells, and these contaminating cells acted in concert with CD4+ Th cells to modulate the development of CD8+ memory T cells. The possible roles of Treg and Th17 cells in tumor progression will be discussed later. In general, Treg cells suppress immune responses to induce immuno tolerance at tumor sites [67], while Th17 cells are evoked in conjunction with acute inXammation and are linked to smoldering inXammation around the tumor lesion to promote tumor incidence and growth [68]. The functions of CD4+ Th cells should be deWned by discounting these Treg/Th17 eVector functions.

The CD4+ Th cells consist of the Th1 and Th2 T cell subsets, based on their distinct cytokine secretion proWles. CD4+ Th1 cells produce cytokines IL-2 and IFN-•. The latter is produced by Th0 (naive T) cells after IL-12 from mDCs stimulate the expression of Stat1 and subsequently that of T-bet, a master transcription factor in Th1 cells [69]. The TICAM-1 pathway in mDCs may contribute to Th1 polarization by preferentially inducing IL-12p40 [55, 60, 70]. CD4+ Th1 cells then provide cytokines for CD8+ T cells and synergistic activation of mDCs, which are essential for CD8+ T cell proliferation and function [71]. IL12p40 is a cytokine that is induced by VAK, which connects with the N- and C-terminal regions of TICAM-1 (Fig. 2b).

In contrast, some TLR ligands may promote the diVerentiation of CD4+ Th2 cells. IL-4 produced by basophils, eosinophils and NKT cells initiates Stat6 signaling, leading to the expression of GATA-3, which is a master transcription factor in Th2 cells [71]. Participation of TICAM-1 in Th2 polarization has been reported [72] but not conWrmed by another group [73]. Several attempts have been made to establish CD4+ T cell clones from tumor-inWltrating T cells. The results indicated that most CD4+ T cell clones are Th1 eVectors that secrete IFN-• and IL-12, but not IL-4 [74].

Th17 cells

IL-17-producing T (Th17) cells are a distinct lineage within the general category of CD4+ Th cells, and secrete a unique set of cytokines, i.e., IL-17 [75, 76]. TGF-• and IL-6 produced by tumor cells, Treg cells and APCs activate the TGF-• and Stat3 signaling pathways, leading to the expression of ROR•t, a critical transcription factor for Th17 cells [77]. Th17 cells were Wrst identiWed as a new CD4+ T cell subset consisting of self-reactive CD4+ Th1 cells. These

cells were later associated with the pathogenesis of many autoimmune diseases [75, 76]. The role of Th17 cells in cancer is less deWned than that of Th1 cells. Nonetheless, both IL-17 and IL-23 have been identiWed in cancer tissues [78], suggesting that Th17 cells together with proinXammatory cytokines may provide an environment favorable for cancer development or invasion. We recently showed that elevated lactic acid in cancer tissues and macrophages in response to TLR stimuli play a key role in IL-23 induction in mDCs or tumor-associated macrophages and help inducing Th17 cells in cancerous environments [79]. Thus, the induction of both IL-23 and IL12p40 by TICAM-1 may be crucial for Th17 stimulation in mDCs. Th17-mediated development of autoimmune disease is constrained by TICAM-1-dependent type I IFN production and its downstream signaling pathway [80]. However, the TICAM-1 region in mDCs that participates in Th17 development is unknown. Th17 cells might play certain roles in tumor progression.

Treg cells

CD4+ Treg cells have been identiWed as a small subset of the T cell population. Several subpopulations of Treg cells have been reported. Naturally occurring CD4+/CD25+ Treg cells together with other CD4+ Treg cells, including CD4+/CD25 ¡ Treg, T•-1 and/or Th3 cells, are involved in T cells regulation [81]. T•-1 cells secrete IFN-• and IL-10, while Th3 cells secrete high levels of TGF-•, IL-4 and IL-10. Foxp3 has been shown to be a speciWc marker of CD4+ Treg cells in both mice and humans [82, 83]. Its expression is highly restricted to the subset of Treg cells and is correlated with immunosuppressor activity, irrespective of CD25 expression.

CD4+ Treg cells can suppress host immune responses to a great extent and induce self-tolerance. Thus, despite their protective role in autoimmune diseases, these cells have inhibitory eVects on cancer immunotherapy and anti-infectious responses [84]. That is, malignant tumors tend to progress more rapidly in a Treg-dominant environment. Recent studies have shown that the proportion of CD4+/CD25+ Treg cells was elevated in the total CD4+ T cell population in several diVerent human cancers, including lung, breast and ovarian tumors [85, 86]. Ag-speciWc CD4+ Treg cells are situated at tumor sites, and these cells suppress the proliferation of naïve CD4+ Th cells upon activation by tumor-speciWc Ags [87]. TLR8 regulates CD4+ Treg function by sensing RNA in Treg cells: adoptive transfer of TLR8 ligand-stimulated Treg cells into tumor-bearing mice enhanced antitumor immunity [88]. Other TLR signaling may be associated with T and mDC functions that are suppressed by tumor-inWltrating •• T cells [89]. Naturally occurring Treg cells require the TICAM-1 pathway in Treg and mDCs for migration to inXamed nests (Fig. 2b), where the MyD88 pathway would restrain their suppressive functions [90]. CD8(+) DEC-205/CD205(+) DCs, but not the CD8(¡) DCs, induce functional Foxp3(+) Treg from Foxp3(¡) precursors in the presence of low doses of Ag [91]. Subsequent inXammatory Th1-type immunity is modulated by induced Treg cells, which also require the TICAM-1 pathway in mDCs [92]. Treg cells inWltrate the tumor mass and exert immunosuppressive eVects that promote tumor progression.

Regulation of TICAM-1 as well as the MyD88 pathway in mDCs may down-regulate Treg in cancer patients [93]. Treg induction is sustained by mDCs with lower maturation stage [94] and what region of TICAM-1 participates in Treg induction remains unknown.

Extrinsic versus intrinsic inXammation for danger signal

PAMPs usually trigger initial or early inXammation around tumors and immune cells in an extrinsic fashion. When tumor cells are damaged through extrinsic inXammation, the destructed cells release cytosolic and nuclear constituents. InXammation is also promoted by these intrinsic nuclear products including HMGB1, uric acids, S100 proteins, cathelicidins, ATP/adenosine and other nucleosomal proteins [95–98]. These molecules are derivatives of nucleic acids or often have DNA/RNA-binding domains. RNA, DNA and other nucleic acids of host origin also act as danger signals [1, 99]. They are released from damaged host cells or tumors and cause long-lasting inXammation [1, 99]. Recently, they have been named danger-associated molecular pattern (DAMP) or alarmin. Since tumor cells frequently undergo cell death by either apoptosis or necrosis, many cytosolic or nuclear factors are liberated from tumor nests. Tumor progression and reciprocal inXammation involve complicated episodes. We could promote tumor damage followed by DAMP liberation by radiation and/or chemotherapy [100, 101]. Recent reviews infer that electrochemotherapy (ECT) and CpG ODN administration to cancer patients synergistically induce a signiWcant increase of the local eVect and a systemic T-dependent antitumor response [100], and that some chemotherapeutic agents with immunostimulatory capacity may facilitate establishing combined chemo-immunotherapy strategies [101]. We should like to clarify these tumor-associated events and responses at a molecular level in order to develop appropriate strategies for the regulation for immune systems in cancer patients. Elucidation of the nucleic acid-recognition systems is essentially required for this purpose. Fundamental issues presented here would hopefully be useful for the development of cancer immunotherapy.

Perspectives

Cancer is a condition in which many immune-related cells form a network in concert with tumor cells. Immune aberrance is an alternative result of tumor growth. APCs and tumor cells exhibit a tight response to innate immune stimulation to alter the balance of tumor tolerance [102].

Cancer stem cells are believed to generate sibling cancer cells. These stem cells are usually vulnerable to irradiation, and their maintenance relies heavily on the gene repair system. It is not known what kinds of RNA sensors and their signaling pathways these stem cells are equipped with [103]. The events that occur in tumor and immune systems upon stem cell modulation by RNA require further study.

Vascular endothelial cells in solid tumors are cytogenetically abnormal. Unlike normal endothelial cells which remain diploid in long-term culture, the aneuploidy of tumor endothelial cells is exacerbated in culture. Tumor-associated endothelial cells upregulate many genes including the epidermal growth factor receptor (EGFR) gene. Accordingly, these cells are highly sensitive to EGF. Endothelial cells usually have a stock of surface-expressed TLR3, which can sense a small RNA duplex structure [104]. Targeting of tumor endothelial cells by immune eVector cells may be a possible therapeutic strategy in anti-angiogenic therapy.

In immunological terms, our trials were aimed at elucidating the mechanisms by which mDCs select the mode of activation for various eVectors. Results from studies on the dsRNA recognition system, indicated that the properties of PAMP and repertoires of host receptors critically aVect these processes. Another issue is how most eVective eVector cells are induced in a case-dependent manner for tumor remission in patients. This review provides guidelines for the development of speciWc eVector cells by selecting dsRNA receptors. Current knowledge on the TICAM-1 pathway could be directly applied to cancer immunotherapy.

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References

1. Kono H, Rock KL (2008) How dying cells alert the immune system to danger. Nat Rev Immunol 8:279–289 (review)
2. Sheu BC, Chang WC, Cheng CY, Lin HH, Chang DY, Huang SC (2008) Cytokine regulation networks in the cancer microenvironment. Front Biosci 13:6255–6268 (review)
3. Melief CJM (2008) Cancer immunotherapy by dendritic cells. Immunity 29:372–383 (review)
4. Rosenberg SA, Yang JC, Restifo NP (2004) Cancer immunotherapy: moving beyond current vaccines. Nat Med 10:909–915 (review)
5. Steinman RM, Hemmi H (2006) Dendritic cells: translating innate to adaptive immunity. Curr Top Microbiol Immunol 311:17–58 (review)
6. Matsumoto M, Seya T (2008) TLR3: interferon induction by double-stranded RNA including poly(I:C). Adv Drug Deliv Rev 60:805–812 (review)
7. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413:732–738
8. Matsuo A, Oshiumi H, Tsujita T, Mitani H, Kasai H, Yoshimizu M, Matsumoto M, Seya T (2008) Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from birnaviruses. J Immunol 181:3474–3485
9. Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T (2004) The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 5:730–737
10. Tanaka H, Samuel CE (1994) Mechanism of interferon action: structure of the mouse PKR gene encoding the interferon-inducible RNA-dependent protein kinase. Proc Natl Acad Sci USA 91:7995–7999
11. Kanneganti TD, Body-Malapel M, Amer A, Park JH, WhitWeld J, Franchi L, Taraporewala ZF, Miller D, Patton JT, Inohara N, Núñez G (2006) Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. J Biol Chem 281:36560–36568
12. Kanneganti TD, Ozören N, Body-Malapel M, Amer A, Park JH, Franchi L, WhitWeld J, Barchet W, Colonna M, Vandenabeele P, Bertin J, Coyle A, Grant EP, Akira S, Núñez G (2006) Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. Nature 440:233–236
13. Malathi K, Dong B, Gale M Jr, Silverman RH (2007) Small self-RNA generated by RNase L ampliWes antiviral innate immunity. Nature 448:816–819
14. Wang RF, Miyahara Y, Wang HY (2008) Toll-like receptors and immune regulation: implications for cancer therapy. Oncogene 27:181–189 (review)
15. Seya T, Hazeki K, Inoue N, Matsumoto M. (2009) Pattern-recognition confers driving various cellular eVectors on dendritic cells. Cancer Sci (in press) (review)
16. Johnson CL, Gale M Jr (2006) CARD games between virus and host get a new player. Trends Immunol 27:1–4 (review)
17. Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, Yamamoto A, Seya T (2003) Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol 171:3154–3162
18. Sasai M, Shingai M, Funami K, Yoneyama M, Fujita T, Matsumoto M, Seya T (2006) NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in type I IFN induction. J Immunol 177:8676–8683
19. Ryzhakov G, Randow F (2007) SINTBAD, a novel component of innate antiviral immunity, shares a TBK1-binding domain with NAP1 and TANK. EMBO J 26:3180–3190
20. Jounai N, Takeshita F, Kobiyama K, Sawano A, Miyawaki A, Xin KQ, Ishii KJ, Kawai T, Akira S, Suzuki K, Okuda K (2007) The Atg5 Atg12 conjugate associates with innate antiviral immune responses. Proc Natl Acad Sci USA 104:14050–14055

21. Maire M, Parent R, Morand AL, Alotte C, Trépo C, Durantel D, Petit MA (2008) Characterization of the double-stranded RNA responses in human liver progenitor cells. Biochem Biophys Res Commun 368:556–562
22. Ermolaeva MA, Michallet MC, Papadopoulou N, Utermöhlen O, Kranidioti K, Kollias G, Tschopp J, Pasparakis M (2008) Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inXammatory responses. Nat Immunol 9:1037–1046
23. Matsumoto M, Kikkawa S, Kohase M, Miyake K, Seya T (2002) Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. Biochem Biophys Res Commun 293:1364–1369
24. Honda K, Taniguchi T (2006) IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol 6:644–658 (review)
25. Yang YL, Reis LF, Pavlovic J, Aguzzi A, Schäfer R, Kumar A, Williams BR, Aguet M, Weissmann C (1995) DeWcient signaling in mice devoid of double-stranded RNA-dependent protein kinase. EMBO J 14:6095–6106
26. Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale M Jr, Akira S, Yonehara S, Kato A, Fujita T (2005) Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J Immunol 175:2851–2858
27. Pichlmair A, Schulz O, Tan CP, Näslund TI, Liljeström P, Weber F, Reis e Sousa C (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. Science 314:997–1001
28. Hornung V, Ellegast J, Kim S, Brzózka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G (2006) 5•-Triphosphate RNA is the ligand for RIG-I. Science 314:994–997
29. Saito T, Owen DM, Jiang F, Marcotrigiano J, Gale M Jr (2008) Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. Nature 454:523–527
30. Absher M, Stinebring WR (1969) Toxic properties of a synthetic double-stranded RNA. Endotoxin-like properties of poly I. poly C, an interferon stimulator. Nature 223:715–717
31. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S (2006) DiVerential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441:101–105
32. O'Neill LA, Bowie AG (2007) The family of Wve: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol 7:353–364 (review)
33. Häcker H, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG, Kamps MP, Raz E, Wagner H, Häcker G, Mann M, Karin M (2006) SpeciWcity in Toll-like receptor signalling through distinct eVector functions of TRAF3 and TRAF6. Nature 439:204–207
34. Chen NJ, Chio II, Lin WJ, Duncan G, Chau H, Katz D, Huang HL, Pike KA, Hao Z, Su YW, Yamamoto K, de Pooter RF, Zúñiga-PXücker JC, Wakeham A, Yeh WC, Mak TW (2008) Beyond tumor necrosis factor receptor: TRADD signaling in toll-like receptors. Proc Natl Acad Sci USA 105:12429–12434
35. Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, Tschopp J (2004) RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat Immunol 5:503–507
36. Vanden Berghe T, van Loo G, Saelens X, Van Gurp M, Brouckaert G, Kalai M, Declercq W, Vandenabeele P (2004) DiVerential signaling to apoptotic and necrotic cell death by Fas-associated death domain protein FADD. J Biol Chem 279:7925–7933
37. Meylan E, Tschopp J, Karin M (2006) Intracellular pattern recognition receptors in the host response. Nature 442:39–44 (review)
38. Honda K, Takaoka A, Taniguchi T (2006) Type I interferon gene induction by the interferon regulatory factor family of transcription factors. Immunity 25:349–360 (review)
39. Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, Seya T (2003) TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. J Biol Chem 278:49751–49762
40. Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, Omori H, Noda T, Yamamoto N, Komatsu M, Tanaka K, Kawai T, Tsujimura T, Takeuchi O, Yoshimori T, Akira S (2008) Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 456:264–268
41. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T (2003) TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat Immunol 4:161–167
42. Seya T, Matsumoto M, Ebihara T, Oshiumi H. (2003) Functional evolution of the TICAM-1 (TRIF) pathway for extrinsic RNA sensing. Immunol Rev (in press) (review)
43. Funami K, Sasai M, Ohba Y, Oshiumi H, Seya T, Matsumoto M (2007) Spatiotemporal mobilization of Toll/IL-1 receptor domain-containing adaptor molecule-1 in response to dsRNA. J Immunol 179:6867–6872
44. Funami K, Sasai M, Oshiumi H, Seya T, Matsumoto M (2008) Homo-oligomerization is essential for Toll/interleukin-1 receptor domain-containing adaptor molecule-1-mediated NF-kappaB and interferon regulatory factor-3 activation. J Biol Chem 283:18283–18291
45. Michallet MC, Meylan E, Ermolaeva MA, Vazquez J, Rebsamen M, Curran J, Poeck H, Bscheider M, Hartmann G, König M, Kalinke U, Pasparakis M, Tschopp J (2008) TRADD protein is an essential component of the RIG-like helicase antiviral pathway. Immunity 28:651–661
46. Foy E, Li K, Sumpter R Jr, Loo YM, Johnson CL, Wang C, Fish PM, Yoneyama M, Fujita T, Lemon SM, Gale M Jr (2005) Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. Proc Natl Acad Sci USA 102:2986–2991
47. Rebsamen M, Meylan E, Curran J, Tschopp J (2008) The antiviral adaptor proteins Cardif and Trif are processed and inactivated by caspases. Cell Death DiVer 15:1804–1811
48. Xu Y, Kim SO, Li Y, Han J (2006) Autophagy contributes to caspase-independent macrophage cell death. J Biol Chem 281:19179–19187
49. Shi CS, Kehrl JH (2008) MyD88 and Trif target Beclin 1 to trigger autophagy in macrophages. J Biol Chem 283:33175–33182
50. Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T (2006) TLR3 can directly trigger apoptosis in human cancer cells. J Immunol 176:4894–4901
51. Zhou R, Wei H, Sun R, Tian Z (2007) Recognition of double-stranded RNA by TLR3 induces severe small intestinal injury in mice. J Immunol 178:4548–4556
52. Han KJ, Su X, Xu LG, Bin LH, Zhang J, Shu HB (2004) Mechanisms of the TRIF-induced interferon-stimulated response element and NF-kappaB activation and apoptosis pathways. J Biol Chem 279:15652–15661
53. McAllister CS, Samuel CE. (2008) Protein kinase PKR enhances the induction of interferon-beta and apoptosis mediated by cytoplasmic RNA sensors. J Biol Chem Nov 20. [Epub ahead of print]
54. Paone A, Starace D, Galli R, Padula F, De Cesaris P, Filippini A, Ziparo E, Riccioli A (2008) Toll-like receptor 3 triggers apoptosis of human prostate cancer cells through a PKC-alpha-dependent mechanism. Carcinogenesis 29:1334–1342

12 3

55. Takeuchi O, Akira S (2007) Recognition of viruses by innate immunity. Immunol Rev 220:214–224 (review)
56. Lin ML, Zhan Y, Villadangos JA, Lew AM (2008) The cell biology of cross-presentation and the role of dendritic cell subsets. Immunol Cell Biol 86:353–362 (review)
57. Akazawa T, Masuda H, Saeki Y, Matsumoto M, Takeda K, Tsujimura K, Kuzushima K, Takahashi T, Azuma I, Akira S, Toyoshima K, Seya T (2004) Adjuvant-mediated tumor regression and tumor-speciWc cytotoxic response are impaired in MyD88-deWcient mice. Cancer Res 64:757–764
58. Shen H, Tesar BM, Walker WE, Goldstein DR (2008) Dual signaling of MyD88 and TRIF is critical for maximal TLR4-induced dendritic cell maturation. J Immunol 181:1849–1858
59. Ebihara T, Matsumoto M, Seya T (2009) NK cell licensing by dendritic cell in RNA virus infection. Curr Immunol Rev (in press) (review)
60. Akazawa T, Ebihara T, Okuno M, Okuda Y, Shingai M, Tsujimura K, Takahashi T, Ikawa M, Okabe M, Inoue N, Okamoto-Tanaka M, Ishizaki H, Miyoshi J, Matsumoto M, Seya T (2007) Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. Proc Natl Acad Sci USA 104:252–257
61. Janssen EM, Lemmens EE, Wolfe T, Christen U, Von Herrath MG, Schoenberger SP (2003) CD4(+) T cells are required for secondary expansion and memory in CD8(+) T lymphocytes. Nature 421:852–856
62. Shedlock DJ, Shen H (2003) Requirement for CD4 T cell help in generating functional CD8T cell memory. Science 300:337–339
63. Sun JC, Bevan MJ (2003) Defective CD8 T cell memory following acute infection without CD4 T cell help. Science 300:339–342
64. Kumar H, Koyama S, Ishii KJ, Kawai T, Akira S (2008) Cutting edge: cooperation of IPS-1- and TRIF-dependent pathways in polyIC-enhanced antibody production and cytotoxic T cell responses. J Immunol 180:683–687
65. Hou B, Reizis B, DeFranco AL (2008) Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. Immunity 29:272–282
66. Hamilton SE, Wolkers MC, Schoenberger SP, Jameson SC (2006) The generation of protective memory-like CD8+ T cells during homeostatic proliferation requires CD4+ T cells. Nat Immunol 7:475–481
67. Yamaguchi T, Hirota K, Nagahama K, Ohkawa K, Takahashi T, Nomura T, Sakaguchi S (2007) Control of immune responses by antigen-speciWc regulatory T cells expressing the folate receptor. Immunity 27:145–159
68. Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, Basham B, McClanahan T, Kastelein RA, Oft M (2006) IL-23 promotes tumour incidence and growth. Nature 442:461–465
69. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH (2000) A novel transcription factor T-bet, directs Th1 lineage commitment. Cell 100:655–669
70. Bohnenkamp HR, Papazisis KT, Burchell JM, Taylor-Papadimitriou J (2007) Synergism of Toll-like receptor-induced interleukin-12p70 secretion by monocyte-derived dendritic cells is mediated through p38 MAPK and lowers the threshold of T-helper cell type 1 responses. Cell Immunol 247:72–84
71. Kuo CT, Leiden JM (1999) Transcriptional regulation of T-lymphocyte development and function. Annu Rev Immunol 17:149–187 (review)
72. Pasare C, Medzhitov R (2005) Control of B-cell responses by Toll-like receptors. Nature 438:364–368
73. Gavin AL, Hoebe K, Duong B, Ota T, Martin C, Beutler B, Nemazee D (2006) Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. Science 314:1936–1938
74. Mami-Chouaib F, Echchakir H, Dorothée G, Vergnon I, Chouaib S (2002) Antitumor cytotoxic T-lymphocyte response in human lung carcinoma: identiWcation of a tumor-associated antigen. Immunol Rev 188:114–121 (review)
75. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT (2005) Interleukin 17-producing CD4+ eVector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 6:1123–1132
76. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, Dong C (2005) A distinct lineage of CD4 T cells regulates tissue inXammation by producing interleukin 17. Nat Immunol 6:1133–1141
77. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR (2006) The orphan nuclear receptor RORgammat directs the diVerentiation program of proinXammatory IL-17(+) T helper cells. Cell 126:1121–1133
78. Langowski JL, Kastelein RA, Oft M (2007) Swords into plowshares: IL-23 repurposes tumor immune surveillance. Trends Immunol 28:207–212 (review)
79. Shime H, Yabu M, Akazawa T, Kodama K, Matsumoto M, Seya T, Inoue N (2008) Tumor-secreted lactic acid promotes IL-23/IL-17 proinXammatory pathway. J Immunol 180:7175–7183
80. Guo B, Chang EY, Cheng G (2008) The type I IFN induction pathway constrains Th17-mediated autoimmune inXammation in mice. J Clin Invest 118:1680–1690
81. Francois Bach J (2003) Regulatory T cells under scrutiny. Nat Rev Immunol 3:189–198 (review)
82. Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. Science 299:1057–1061
83. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY (2005) Regulatory T cell lineage speciWcation by the forkhead transcription factor foxp3. Immunity 22:329–341
84. Shevach EM (2002) CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2:389–400 (review)
85. Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, Rubin SC, Kaiser LR, June CH (2001) Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer Res 61:4766–4772
86. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS, Linehan DC (2002) Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J Immunol 169:2756–2761
87. Wang HY, LEE DA, Peng G, Guo Z, Li Y, Kiniwa Y, Shevach EM, Wang RF (2004) Tumor-speciWc human CD4+ regulatory T cells and their ligands: implication for immunotherapy. Immunity 20:107–118
88. Peng G, Guo Z, Kiniwa Y, Voo KS, Peng W, Fu T, Wang DY, Li Y, Wang HY, Wang RF (2005) Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. Science 309:1380–1384
89. Peng G, Wang HY, Peng W, Kiniwa Y, Seo KH, Wang RF (2007) Tumor-inWltrating gammadelta T cells suppress T and dendritic cell function via mechanisms controlled by a unique toll-like receptor signaling pathway. Immunity 27:334–348
90. Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC (2007) The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science 316:1628–1632
91. Yamazaki S, Dudziak D, Heidkamp GF, Fiorese C, Bonito AJ, Inaba K, Nussenzweig MC, Steinman RM (2008) CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. J Immunol 181:6923–6933
92. Pasare C, Medzhitov R (2003) Toll pathway-dependent blockade of CD4+ CD25+ T cell-mediated suppression by dendritic cells. Science 299:1033–1036
93. Warger T, Osterloh P, Rechtsteiner G, Fassbender M, Heib V, Schmid B, Schmitt E, Schild H, Radsak MP (2006) Synergistic

12 3

activation of dendritic cells by combined Toll-like receptor ligation induces superior CTL responses in vivo. Blood 108:544–550
94. Yamazaki S, Bonito AJ, Spisek R, Dhodapkar M, Inaba K, Steinman RM (2007) Dendritic cells are specialized accessory cells along with TGF- for the diVerentiation of Foxp3+ CD4+ regulatory T cells from peripheral Foxp3 precursors. Blood 110:4293–4302
95. Hiratsuka S, Watanabe A, Sakurai Y, Akashi-Takamura S, Ishibashi S, Miyake K, Shibuya M, Akira S, Aburatani H, Maru Y (2008) The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. Nat Cell Biol 10:1349–1355
96. Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, Abraham E (2004) Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem 279:7370–7377
97. Shi Y, Evans JE, Rock KL (2003) Molecular identiWcation of a danger signal that alerts the immune system to dying cells. Nature 425:516–521
98. Curtiss LK, Tobias PS (2008) Emerging role of toll-like receptors in atherosclerosis. J Lipid Res 2008 Nov 1 [Epub ahead of print]
99. CoVelt SB, Scandurro AB (2008) Tumors sound the alarmin(s). Cancer Res 68:6482–6485 (review)
100. Roux S, Bernat C, Al-Sakere B, Ghiringhelli F, Opolon P, Carpentier AF, Zitvogel L, Mir LM, Robert C (2008) Tumor destruction using electrochemotherapy followed by CpG oligodeoxynucleotide injection induces distant tumor responses. Cancer Immunol Immunother 57:1291–1300 (review)
101. Ménard C, Martin F, Apetoh L, Bouyer F, Ghiringhelli F (2008) Cancer chemotherapy: not only a direct cytotoxic eVect, but also an adjuvant for antitumor immunity. Cancer Immunol Immunother 57:1579–1587 (review)
102. Seya T, Akazawa T, Uehori J, Matsumoto M, Azuma I, Toyoshima K (2003) Role of toll-like receptors and their adaptors in adjuvant immunotherapy for cancer. Anticancer Res 23:4369–4376 (review)
103. Nagai Y, Garrett KP, Ohta S, Bahrun U, Kouro T, Akira S, Takatsu K, Kincade PW (2006) Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. Immunity 24:801–812
104. Kleinman ME, Yamada K, Takeda A, Chandrasekaran V, Nozaki M, BaY JZ, Albuquerque RJ, Yamasaki S, Itaya M, Pan Y, Appukuttan B, Gibbs D, Yang Z, Karikó K, Ambati BK, Wilgus TA, DiPietro LA, Sakurai E, Zhang K, Smith JR, Taylor EW, Ambati J (2008) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature 452:591–597

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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

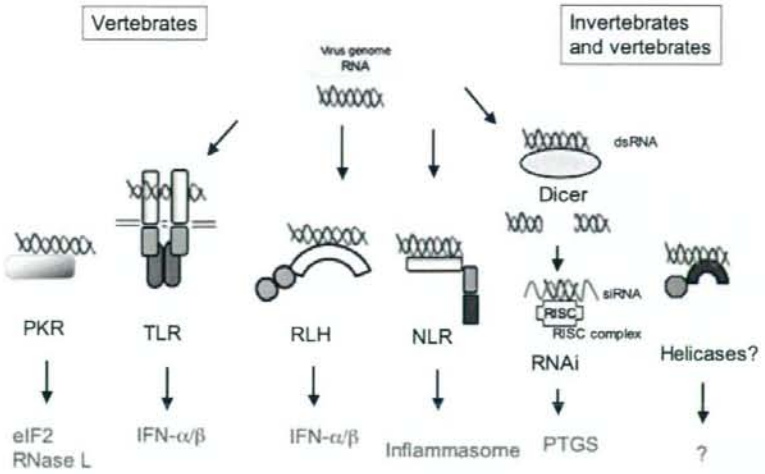


Fig. 1. Various RNA sensors in vertebrates. dsRNA are generated during virus replication. Major RNA sensors in vertebrate cells and their responses on stimulation with dsRNA are indicated. Dicer and RNA-recognizing helicases work even in invertebrates. How dsRNA selects a variety of RNA pattern sensors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

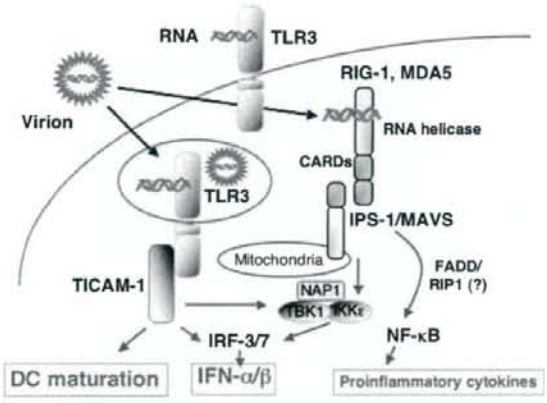
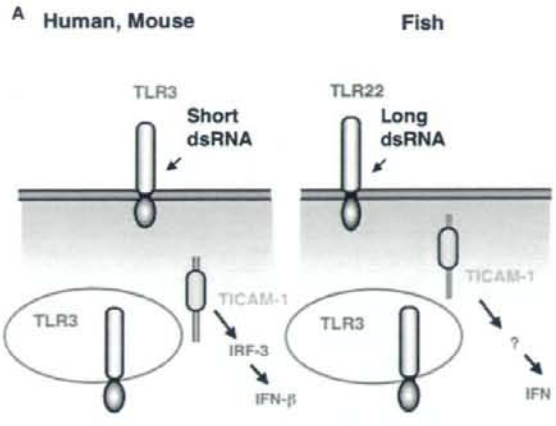


Fig. 2. Cell surface, endosomal and cytoplasmic recognition of dsRNA in mammalian cells. TLR3 is distributed either on the cell surface or in the endosome. Its distribution ratio depends on cell types. RLH (RIG-I and MDA5) reside in the cytoplasm. Adapter molecules, TICAM-1 and MAVS, are localized in 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



B **IFN-inducing receptors in human and fish**

Genes	Human	Fish
TLR3	+	+
RIG-I	+	–
TLR22	–	+
IFN	+	+

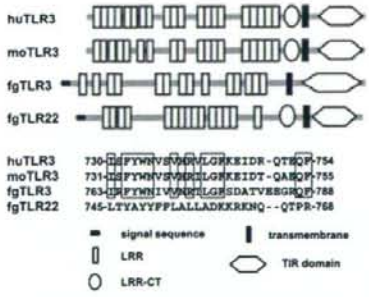


Fig. 3. Different TLRs cover surface dsRNA recognition in fish and mammals. (A) TLR3 and TLR22 in vertebrates. In human and mouse, TLR3 encompasses cell surface and endosomal RNA sensing and induces IRF-3 activation. In fish, two distinct gene products, TLR3 and TLR22, participate in dsRNA sensing. IFN 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.

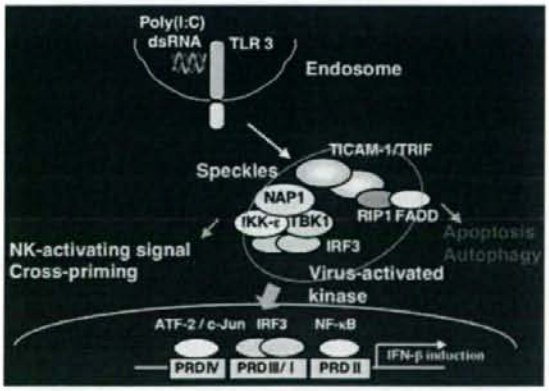


Fig. 4. TICAM-1 is dissociated from TLR3 to form a signaling unit, Speckle. In human cells, TICAM-1 once detached from TLR3 serves as a signaling platform to induce apoptosis, autophagy, NK activation, and cross-priming. TICAM-1 undergoes some modification secondary to complex formation with TLR3 and 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

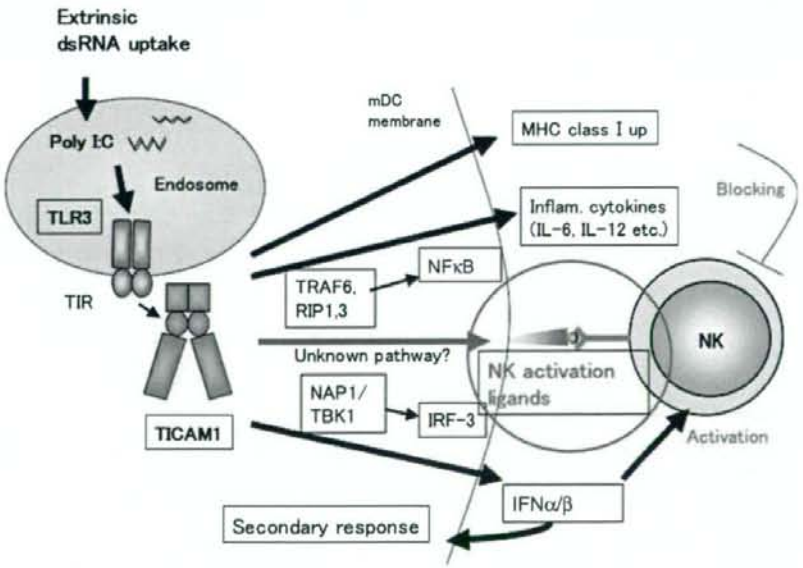


Fig. 5. Mechanism of mDC-NK reciprocal activation induced by dsRNA stimulation of mDCs. TICAM-1 has a crucial role in NK activation driven by polyI:C-stimulated mDCs in human cells. When TLR3 grasps the dsRNA signature in the endosome of mDCs, TICAM-1 in mDCs is activated to evoke a signal pathway reaching 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.

mDC TICAM-1 pathway efficiently links CTL/NK activation by mDCs. Missing the cell surface-specific TLR, TLR22, and conserving ER-resident TLR, TLR3, in mDCs may cause the functional specialization of the TICAM-1 pathway on evoking cellular immunity in mammals. Although the signaling pathway by which type I IFN is induced has been elucidated in each cell type, the exact pathway that drives NK activation or CTL induction by mDCs has not been identified.

Effector induction in transgenic mice with TLR22 for surface dsRNA recognition

Upon transfection of fgTLR22 or fgTLR3 into human or mouse cells, fgTLR22 functions as an RNA sensor for IFN induction in these mammalian cells, suggesting that mice and human TICAM-1 are compatible with fish TLR22 and TLR3 (37). With this finding in mind, we have generated TLR22 transgenic (Tg) mice to test fish TLR22 antiviral function and NK activation in mouse. TLR22 is ubiquitously expressed in all the organs tested in the Tg mice (A. Matsuo, H. Oshiumi, T. Seya, unpublished data). Its expression profile is similar to that in fish, in which endogenous fish TICAM-1 is ubiquitously expressed. PolyI:C or poliovirus were used as type I IFN inducers for *in vitro* mouse embryonic fibroblasts (MEF) stimulation studies. TLR22-expressing MEFs produce high levels of type I IFN within 6 h, a time period during which control MEFs still do not produce type I IFN. Rapid induction and three- to fivefold higher levels of IFN-β in the supernatant are characteristic features of TLR22-expressing MEFs. Similar results were obtained with BMDCs.

The levels of NK activation induced by BMDCs do not differ significantly between TLR22-expressing BMDCs and control BMDCs. We believe that TLR22 differs from TLR3 in its ability to activate cellular immune responses. However, further investigation is necessary to establish the final conclusion.

Virus infection studies were performed on Tg mice using influenza virus and poliovirus in an *in vivo* mouse model (A. Matsuo, H. Oshiumi, T. Seya, unpublished data). Both Tg and control mice died of influenza infection within 7 days. It appeared that TLR22 did not protect mice from influenza. By contrast, Tg mice expressing the poliovirus receptor (PVR) and TLR22 were relatively resistant to poliovirus infection compared with TLR22-negative control PVR-Tg mice. Wildtype mice died within 5 days, but Tg mice survived for a significant longer period. Hence, TLR22 harbors antiviral activity against acute infection of dsRNA or positive-stranded RNA viruses. This TLR22 function is conserved in TLR22-positive cells of Tg mice. We support the interpretation that TLR22 is lost in mammals so that the TLR22 supplement recovers resistance to dsRNA-generating viruses. The summary of this issue on TLR22-Tg mice is illustrated in Fig. 6.

Although cell surface activation of TLR3 or TLR22 may not be associated with induction of cellular immunity, these molecules efficiently suppress acute viral infection by generating type I IFN. Development of the endosomal RNA recognition system in mDCs would be essential in mammals for enhancing the induction of cell-mediated and long-lasting immunity in viral infection. Although to what extent TLR22 participates in the induction of cellular immunity by virus infection remains largely unsettled, fish unequivocally develop the endosomal RNA recognition system involving TLR3. Cell surface RNA recognition by TLR3 exerts some toxic features (7, 53), which may facilitate limited usage of TLR3 on membrane surface. Part of the linking between TLRs and cellular immune responses should have been established before human and fish ancestors diverged.

Prototype of the vertebrate TLR system

The phylogenetic tree of vertebrate TLR family members strongly supports the notion that non-mammalian vertebrate TLRs emerged during the Cambrian period together with other mammalian TLRs (13, 14, 36, 49); thus, the human ancestor probably possessed both contemporary TLR subsets and those of non-mammalian vertebrates. Based on our knowledge of the functional coverage of vertebrate TLR family members, the expected TLR subsets that the vertebrate

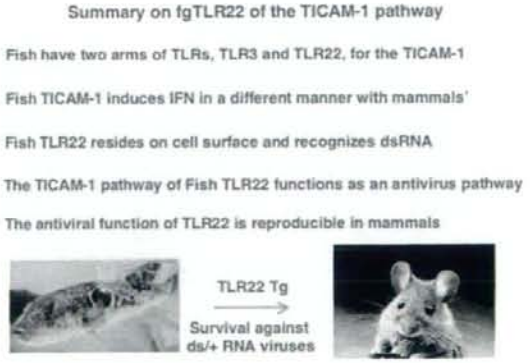


Fig. 6. Summary on fgTLR22 of the TICAM-1 pathway. (i) Fish have two arms of TLRs, TLR3, and TLR22, for the TICAM-1. (ii) Fish TICAM-1 induces IFN in a different manner with mammals. (iii) Fish TLR22 resides on cell surface and recognizes dsRNA. (iv) The TICAM-1 pathway of fish TLR22 functions as an antivirus pathway. (v) The antiviral function of TLR22 is reproducible in mammals.

common ancestor would have possessed would include at least the following 10 TLR members: TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9, TLR21, TLR21, and TLR22 (13) (*Table* 1). Prior to the evolution of mammals, gene duplications would have occurred, especially in TLR2 subfamily members. Furthermore, some TLR genes were lost in some lineages, although the reason remains unknown. For example, TLR21 was diminished in the mammalian lineage, and TLR22 was lost when the mammalian ancestor began to live on land (36). Why did our human ancestor lose TLR21 and TLR22 during evolution? There are two possible explanations. First, mammals need to recognize patterns in the endosome to link the acquired responses so that non-mammalian TLRs present on the cell surface would become dispensable in the innate system. This scenario is conceivable, because the acquired system in mammals is far more sophisticated than that of teleosts. Second, the mammalian lineage happened to lose the non-mammalian TLRs. This observation is not surprising because loss of genes, which are useful for the descendant, has occurred occasionally during vertebrate evolution. For example, the vertebrate ancestor probably possessed broader spectral opsin genes for light sensing, keener auditory sensors for sound hearing, and more olfactory genes for smell sensing than humans, but the mammalian ancestor lost these outstanding genes since their divergence from reptiles (54); thus, many mammalian species are less sensitive to distal light wavelength of light, high frequency of sound, and faint smell than other non-mammalian vertebrates. If mammals had successfully reproduced TLR22 in their genomes, innate immunity in humans would have been stronger. Optional environmental pressure by pathogens may have led to the divergence of the immune system, resulting in variations. In any case, TLRs linked cellular immunity a long time ago: a common ancestor of fish and human already had a prototype. Based on this view, it appears that our immune system is not ideal but is just an example of how infections with certain pathogens have been prevented over a long time period.

References

1. Medzhitov R, Janeway CA Jr. Innate immunity: the virtues of a nonclonal system of recognition. Cell 1997;**91**:295–298.
2. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature 1997;**388**:394–397.
3. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;**124**:783–801.
4. Yoneyama M, Fujita T. Function of RIG-I-like receptors in antiviral innate immunity. J Biol Chem 2007;**282**:15315–15318.
5. Matsumoto M, Seya T. TLR3: interferon induction by double-stranded RNA including poly(I:C). Adv Drug Deliv Rev 2008;**60**: 805–812.
6. Carter WA, Pitha PM, Marshall LW, Tazawa I, Tazawa S, Ts'o PO. Structural requirements of the rI n–rC n complex for induction of human interferon. J Mol Biol 1972;**70**:567–587.
7. Absher M, Stinebring WR. Toxic properties of a synthetic double-stranded RNA. Endotoxin-like properties of poly I:poly C, an interferon stimulator. Nature 1969; **223**:715–717.
8. Sasai M, Shingai M, Funami K, Yoneyama M, Fujita T, Matsumoto M, Seya T. NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in type I IFN induction. J Immunol 2006;**177**: 8676–8683.
9. Honda K, Taniguchi T. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol 2006;**6**:644–658.
10. Hoshino K, Kaisho T. Nucleic acid sensing Toll-like receptors in dendritic cells. Curr Opin Immunol 2008;**20**:408–413.
11. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004;**5**:987–995.
12. Reis e Sousa C. Dendritic cells in a mature age. Nat Rev Immunol 2006;**6**: 476–483.
13. Oshiumi H, Tsujita T, Shida K, Matsumoto M, Ikeo K, Seya T. Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome. Immunogenetics 2003;**54**: 791–800.
14. Roach JC, et al. The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci USA 2005;**102**:9577–9582.
15. Baoprasertkul P, Peatman E, Somridhivej B, Liu Z. Toll-like receptor 3 and TICAM genes in catfish: species-specific expression profiles following infection with *Edwardsiella ictaluri*. Immunogenetics 2006;**58**:817–830.
16. Sullivan C, Postlethwait JH, Lage CR, Millard PJ, Kim CH. Evidence for evolving Toll-IL-1 receptor-containing adaptor molecule function in vertebrates. J Immunol 2007; **178**:4517–4527.
17. Yoneyama M, Onomoto K, Fujita T. Cytoplasmic recognition of RNA. Adv Drug Deliv Rev 2008;**60**:841–846.
18. Matsumoto M, et al. Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol 2003;**171**:3154–3162.
19. Ebihara T, Shingai M, Matsumoto M, Wakita T, Seya T. Hepatitis C virus-infected hepatocytes extrinsically modulate dendritic cell maturation to activate T cells and natural killer cells. Hepatology 2008;**48**: 48–58.
20. Fan S, et al. Zebrafish TRIF, a Golgi-localized protein, participates in IFN induction and NF-kappaB activation. J Immunol 2008;**180**:5373–5383.
21. Matsumoto M, Kikkawa S, Kohase M, Miyake K, Seya T. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. Biochem Biophys Res Commun 2002;**293**:1364–1369.
22. Funami K, Matsumoto M, Oshiumi H, Akazawa T, Yamamoto A, Seya T. The cytoplasmic 'linker region' in Toll-like receptor 3 controls receptor localization and signaling. Int Immunol 2004;**16**:1143–1154.
23. de Bouteiller O, et al. Recognition of double-stranded RNA by human toll-like receptor 3 and downstream receptor signaling requires multimerization and an acidic pH. J Biol Chem 2005;**280**:38133–38145.
24. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat Immunol 2003;**4**:161–167.

25. Yamamoto M, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003;**301**: 640–643.
26. Häcker H, et al. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature 2006;**439**:204–207.
27. Oganesyan G, et al. Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. Nature 2006;**439**:208–211.
28. Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. Science 2003;**300**: 1148–1151.
29. Fitzgerald KA, et al. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003;**4**:491–496.
30. Akazawa T, et al. Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. Proc Natl Acad Sci USA 2007;**104**:252–257.
31. Schulz O, et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature 2005;**433**:887–892.
32. Meylan E, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, Tschopp J. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. Nat Immunol 2004;**5**:503–507.
33. Salem ML, Kadima AN, Cole DJ, Gillanders WE. Defining the antigen-specific T-cell response to vaccination and poly(I:C)/TLR3 signaling: evidence of enhanced primary and memory CD8 T-cell responses and antitumor immunity. J Immunother 2005;**28**: 220–228.
34. Sivori S, et al. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. Proc Natl Acad Sci USA 2004;**101**: 10116–10121.
35. Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T. TLR3 can directly trigger apoptosis in human cancer cells. J Immunol 2006;**176**:4894–4901.
36. Oshiumi H, Matsuo A, Matsumoto M, Seya T. Pan-vertebrate Toll-like receptors during evolution. Curr Genomics 2008;**9**:488–493.
37. Matsuo A, et al. Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from birnaviruses. J Immunol 2008;**181**:3474–3485.
38. Funami K, Sasai M, Ohba Y, Oshiumi H, Seya T, Matsumoto M. Spatiotemporal mobilization of TICAM-1 in response to dsRNA. J Immunol 2007;**179**: 6827–6830.
39. Funami K, Sasai M, Oshiumi H, Seya T, Matsumoto M. Homo-oligomerization is essential for Toll/IL-1 receptor domain containing adaptor molecule-1 signaling. J Biol Chem 2008;**283**:18283–18291.
40. Bergan V, Steinsvik S, Xu H, Kileng Ø, Robertsen B. Promoters of type I interferon genes from Atlantic salmon contain two main regulatory regions. FEBS J 2006; **273**:3893–3906.
41. Rudd BD, et al. Deletion of TLR3 alters the pulmonary immune environment and mucus production during respiratory syncytial virus infection. J Immunol 2006; **176**:1937–1942.
42. Harada K, et al. Innate immune response to double-stranded RNA in biliary epithelial cells is associated with the pathogenesis of biliary atresia. Hepatology 2007;**46**: 1146–1154.
43. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun 2000;**68**:7010–7017.
44. Nakamura MK, et al. Increased expression of TLR3 in human intrahepatic biliary epithelial cells at the site of ductular reaction in primary billiary cirrhosis. Hepatol Intern 2008;**2**:222–230.
45. Phelan PE, Pressley ME, Witten PE, Mellon MT, Blake S, Kim CH. Characterization of snakehead rhabdovirus infection in zebrafish (*Danio rerio*). J Virol 2005;**79**:1842–1852.
46. Nishizawa T, Kinoshita S, Yoshimizu M. An approach for genogrouping of Japanese isolates of aquabirnaviruses in a new genogroup, VII, based on the VP2/NS junction region. J Gen Virol 2005;**86**:1973–1978.
47. Ando T, Suzuki H, Nishimura S, Tanaka T, Hiraishi A, Kikuchi K. Characterization of extracellular RNAs produced by the marine photosynthetic bacterium *Rhodovulum sulfidophilum*. J Biochem 2006;**139**:805–811.
48. Coulibaly F, et al. The birnavirus crystal structure reveals structural relationships among icosahedral viruses. Cell 2005;**120**:761–772.
49. Ishii A, Kawasaki M, Matsumoto M, Tochinai S, Seya T. Phylogenetic and expression analysis of amphibian *Xenopus* Toll-like receptors. Immunogenetics 2007;**59**:281–293.
50. Akazawa T, et al. Tumor immunotherapy using bone marrow-derived dendritic cells overexpressing Toll-like receptor adaptors. FEBS Lett 2007;**581**:3334–3340.
51. Akazawa T, et al. Adjuvant-mediated tumor regression and tumor-specific cytotoxic response are impaired in MyD88-deficient mice. Cancer Res 2004;**64**:757–764.
52. Seya T, Akazawa T, Uehori J, Matsumoto M, Azuma I, Toyoshima K. Role of toll-like receptors and their adaptors in adjuvant immunotherapy for cancer. Anticancer Res 2003;**23**:4369–4376.
53. Zhou R, Wei H, Sun R, Tian Z. Recognition of double-stranded RNA by TLR3 induces severe small intestinal injury in mice. J Immunol 2007;**178**:4548–4556.
54. International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. Nature 2004;**431**:931–945.

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Riplet/RNF135, a RING Finger Protein, Ubiquitinates RIG-I to Promote Interferon-β Induction during the Early Phase of Viral Infection*[S]

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RIG-I (retinoic acid-inducible gene-I), a cytoplasmic RNA helicase, interacts with IPS-1/MAVS/Cardif/VISA, a protein on the outer membrane of mitochondria, to signal the presence of virus-derived RNA and induce type I interferon production. Activation of RIG-I requires the ubiquitin ligase, TRIM25, which mediates lysine 63-linked polyubiquitination of the RIG-I N-terminal CARD-like region. However, how this modification proceeds for activation of IPS-1 by RIG-I remains unclear. Here we identify an alternative factor, Riplet/RNF135, that promotes RIG-I activation independent of TRIM25. The Riplet/RNF135 protein consists of an N-terminal RING finger domain, C-terminal SPRY and PRY motifs, and shows sequence similarity to TRIM25. Immunoprecipitation analyses demonstrated that the C-terminal helicase and repressor domains of RIG-I interact with the Riplet/RNF135 C-terminal region, whereas the CARD-like region of RIG-I is dispensable for this interaction. Riplet/RNF135 promotes lysine 63-linked polyubiquitination of the C-terminal region of RIG-I, modification of which differs from the N-terminal ubiquitination by TRIM25. Overexpression and knockdown analyses revealed that Riplet/RNF135 promotes RIG-I-mediated interferon-β promoter activation and inhibits propagation of the negative-strand RNA virus, vesicular stomatitis virus. Our data suggest that Riplet/RNF135 is a novel factor of the RIG-I pathway that is involved in the evoking of human innate immunity against RNA virus infection, and activates RIG-I through ubiquitination of its C-terminal region. We infer that a variety of RIG-I-ubiquitinating molecular complexes sustain RIG-I activation to modulate RNA virus replication in the cytoplasm.

Cytoplasmic viral RNA sensors induce production of type I interferon (IFN)[2] (1, 2). Representative cytoplasmic sensors, RIG-I-like receptors (RLRs) of RIG-I, MDA5, and LGP2, belong to the DEA(D/H) box RNA helicase family (3–6). RIG-I recognizes the 5′ end triphosphate of the virus RNA genome or double-stranded RNA (6–8) to sense infection by various RNA viruses (3, 5). The RIG-I protein consists of two N-terminal CARD-like domains, an RNA helicase region and a repressor domain (RD) (9). After recognition of positive or negative single-stranded viral RNA, RIG-I interacts with its adaptor molecule IPS-1/MAVS/Cardif/VISA leading to type I IFN production, thereby protecting host cells from amplified viral replication (10–13). However, only a few copies of viral RNAs usually penetrate the cell membrane to enter the cell at an early infection, and these RLRs are barely present in intact as well as early virus-infected cells (6). The early viral RNA recognition facility should be different from that of the late phase when RIG-I protein is abundant in the cytoplasm and easily re-organizes the virus RNAs. What molecular mechanism is responsible for initial sensing of viral RNA thus remains unknown.

Other RLRs, MDA5 and LGP2, are structurally similar to RIG-I in their having the helicase domain (5, 14). However, MDA5 lacks the RD domain although it possesses CARD-like region at the N terminus like RIG-I. LGP2 does not have a CARD-like region but possesses RD at its C terminus (9). RIG-I and MDA5 recognize different kinds of RNA viruses and in some cases play a redundant role in sensing virus infection, such as influenza B (15). In contrast, LGP2 rather negatively regulates virus replication. LGP2 expression suppressed RIG-I or MDA5 signaling (14, 16), and *lgp2* gene disruption conferred high susceptibility to virus infection on mice (4).

Recently, the majority of proteins involved in the type I IFN-inducing system were found ubiquitinated. For example, the tumor necrosis factor receptor-associated family members, TRAF3 and TRAF6, are ubiquitin ligases to induce ubiquitination of proteins and implicated in activation of IFN regulatory factor (IRF) 3 or nuclear factor (NF) κB (13, 17–19). In contrast, a deubiquitinating enzyme, DUBA or A20, suppresses these signals (19, 20). In addition to ubiquitin, ubiquitin-like protein, ISG15, is also conjugated to proteins involved in the IFN-inducing pathway (21, 22). Recent studies have revealed that viral RNA sensors are also ubiquitinated. TRIM25 (ZNF147 or EFP), a member of the ubiquitin-protein isopeptide ligase family, which possesses a RING finger domain, ubiquitinates the

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[2] The abbreviations used are: IFN, interferon; RT, reverse transcription; RLR, RIG-I-like receptor; HA, hemagglutinin; siRNA, small interference; m.o.i., multiplicity of infection; VSV, vesicular stomatitis virus; IRF, IFN regulatory factor; Ub, ubiquitin; ORF, open reading frame; RD, repressor domain.

CARD-like domains of RIG-I thereby facilitating the RIG-I-mediated activation of type I IFN signaling (23, 24), although Shimotohno and co-workers (25) previously reported that TRIM25 (EFP) does not polyubiquitinate the RIG-I CARD-like region as far under their conditions. Expression of TRIM25 increases RIG-I CARD-like region-mediated signaling; however, it remains to be determined whether the activation of full-length RIG-I requires other ubiquitin ligase (23). Another ubiquitin ligase RNF125 mediates lysine 48-linked polyubiquitination of RIG-I, which leads to degradation of RIG-I through the proteasome (25).

Here we examined what molecular complex participates in an early RIG-I-mediated RNA recognition and IFN signaling by yeast two-hybrid screening. Here we detected two novel RING finger proteins that bound to RIG-I, and we found that one, RNF135, facilitated RIG-I-mediated type I IFN induction via ubiquitinating RIG-I. RNF135 plays a crucial role in the RIG-I response to minimal copies of viral RNA, and by binding to the C-terminal helicase and RD regions of RIG-I, RNF135 facilitates RIG-I C-terminal ubiquitination to up-regulate RIG-I-mediated IFN signaling and suppress viral replication. Hence, we renamed it as RNF135 Riplet (RING finger protein leading to RIG-I activation). To our knowledge, this is the first study demonstrating that C-terminal ubiquitination of RIG-I is important for full IFN induction by RIG-I.

EXPERIMENTAL PROCEDURES

Cell Cultures—HEK293 and Vero cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Invitrogen), and HeLa cells were in minimum Eagle's medium with 2 mM L-glutamine and 10% fetal calf serum (JRH Biosciences). HEK293FT cells were maintained in Dulbecco's modified Eagle's high glucose medium containing 10% heat-inactivated fetal calf serum (Invitrogen).

Plasmids—cDNA fragment encoding a C-terminal region of Riplet was isolated by yeast two-hybrid screening using human lung cDNA library. The 5′ region encoding the remaining N-terminal region was amplified by PCR using primers Riplet-F1 and Riplet-R1, and human lung cDNA library was used for its template. Two cDNA fragments, which cover the entire ORF of Riplet, were joined by PCR using primers Riplet-F1, R1, F2, and R2 and then inserted into pCR-blunt vector (Invitrogen). The primers sequences are as follows: F1, GCCTCGAGGCCACCATGGCGGGCCTGGGCCTGGG; R1, CGGCCAGGTCCTGCAGTAGC; F2, GCACCTGCGGAAGAACACGC; and R2, GGGGATCCCACCTTTACTTGCTTTATTATCAGG. The obtained cDNA was cloned into XhoI-NotI restriction sites of pEF-BOS expression vector, and the HA tag was fused at the C-terminal end of Riplet. Riplet-DN (dominant negative) expression vector was constructed by amplifying the relevant Riplet cDNA fragment using the primers Riplet-X-F-C and Riplet-R2 and subcloned into pEF-BOS. The primer sequence of Riplet-X-F-C was as follows: GCTCGAGGCCACCATGCCGCACCTGCGGAAGAACACGC. Riplet-L248fs expression vector was made by deleting 1 base at position 742 by standard PCR-mediated site-directed mutagenesis methods with primers Riplet-L248fs-F and Riplet-L248fs-R as follows: Riplet-L248fs-F, CCAGAGCCACCCTGCATCAGGAGAGCTTCTCGG, and Riplet-L248fs-R, CCGAGAAGCTCTCCTGATGCAGGGTGGCTCTGG. All cloned *RIPLET* cDNA fragments were sequenced, and it was confirmed that there were no mutations. Full-length RIG-I expressing vector, Gal4-IRF-3, Gal4-DBD, and p55 UASG-Luc reporter plasmids were gifts from Dr. T. Fujita (Kyoto University, Kyoto, Japan). p125 luc reporter plasmid was a gift from Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). RIG-I RD expressing vector was made with primers RIG-I RD-F and RIG-I RD-R; the RIG-I dRD cDNA fragment, which encodes ORF of RIG-I from the 1- to 754-amino acid region, was made by using primers RIG-I-(1–754)F and RIG-I-(1–754)R. The obtained cDNA fragments were sequenced, and it was confirmed that there were no mutations caused by PCR. The primers sequences are as follows: RIG-I RD-F, GAT GAT AAA GGT ACC ACC GGT AGC AAG TGC TTC CTT CTG; RIG-I RD-R, AAG GAA GCA CTT GCT ACC GGT GGT ACC TTT ATC ATC ATC ATC; RIG-I-(1–754)F, GC AGA GGA AGA GCA AGA TGA TAT CAG GTC CTC AAT CTT C; and RIG-I-(1–754)R, ATT GAG GAC CTG ATA TCA TCT TGC TCT TCC TCT GCC TC.

Northern Blotting—Human *RIPLET* 1092-bp cDNA fragment (208–1299) was used for the probe for Northern blotting. The Northern blot membranes, human 12-lane MTN blot and MTN blot III, were purchased from Clontech. The homology of human *RIPLET* and *TRIM25* in the probe region was 46%. We used a stringent condition for Northern blotting to exclude the cross-hybridization between the *RIPLET* and *TRIM25* genes. Briefly, the probe was labeled with [α-^{32}P]dCTP using Rediprime II Random Prime labeling system (GE Healthcare). The labeled probe was hybridized to the membrane with ExpressHyb hybridization solution (Clontech) at 68 °C for 1 h. The membrane was washed with washing solution I (2× SSC, 0.05% SDS) for 40 min, and then washed with washing solution II (0.1× SSC, 0.1% SDS) for 40 min. Riplet mRNA bands were detected with x-ray film.

Reporter Gene Analysis—HEK293 cells were transiently transfected in 24-well plates using FuGENE HD (Roche Applied Science) with expression vectors, reporter plasmids, and internal control plasmid coding *Renilla* luciferase. The total amounts of plasmids were normalized with empty vector. For poly(I-C) stimulation, 24 h after transfection, cells were stimulated with medium containing poly(I-C) (50 μg/ml) and DEAE-dextran (0.5 mg/ml) for 1 h, and then the medium was exchanged with normal medium and incubated for an additional 3 h. Cells were lysed with lysis buffer (Promega) and luciferase, and *Renilla* luciferase activities were measured by the dual luciferase assay kit (Promega). Relative luciferase activities were calculated by normalizing luciferase activity by *Renilla* luciferase activity, and dividing the normalized value by control in which only empty vector, reporter, and internal control plasmid were transfected. Values are expressed as mean relative stimulations ± S.D. for a representative experiment, and each was performed three times in duplicate (unless otherwise indicated in the legends).

RNA Interference—Reporter and siRNA (20 nM final concentration) for Riplet or control were transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen) by the standard method described in the manufacturer's protocol. Empty vec-