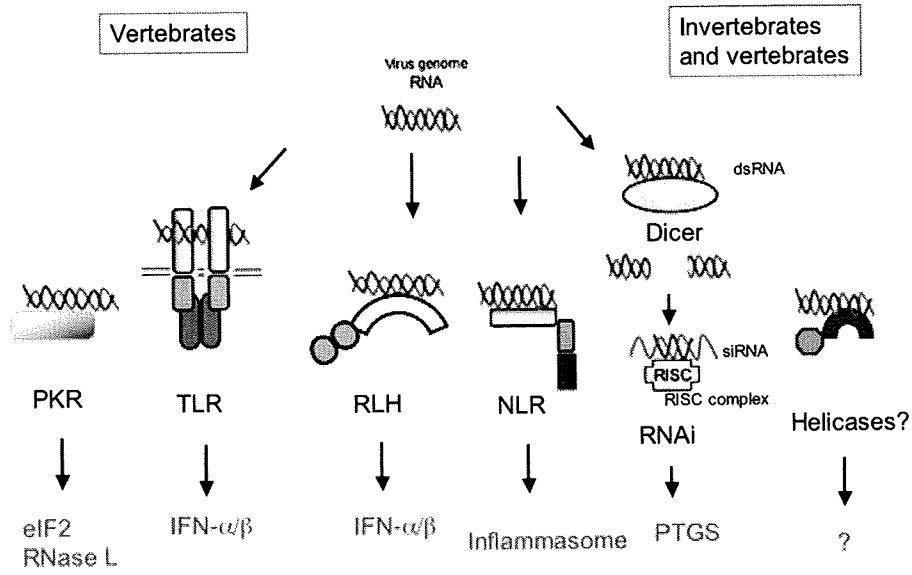


Fig. 1. Various RNA sensors in vertebrates. dsRNA are generated during virus replication. Major RNA sensors in vertebrate cells and their responses on stimulation with dsRNA are indicated. Dicer and RNA-recognizing helicases work even in invertebrates. How dsRNA selects a variety of RNA pattern sensors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

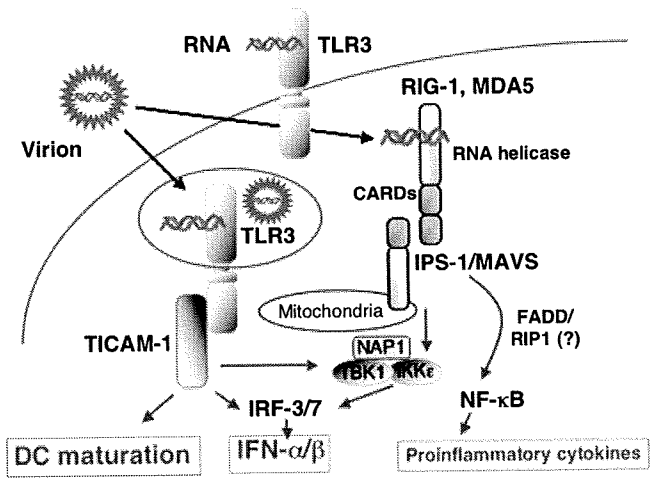
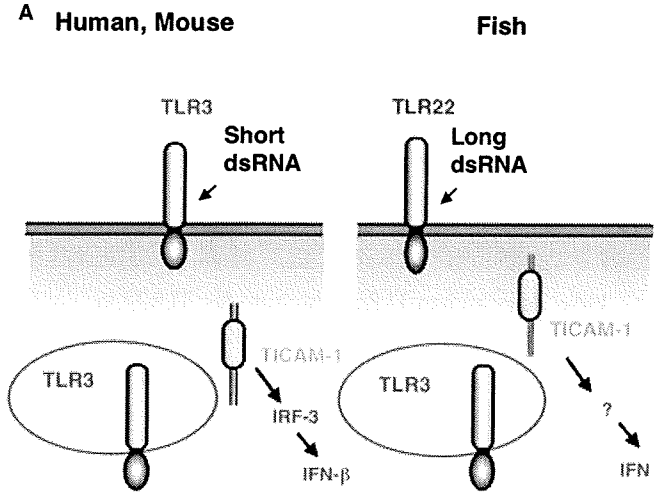


Fig. 2. Cell surface, endosomal and cytoplasmic recognition of dsRNA in mammalian cells. TLR3 is distributed either on the cell surface or in the endosome. Its distribution ratio depends on cell types. RLH (RIG-I and MDA5) reside in the cytoplasm. Adapter molecules, TICAM-1 and MAVS, are localized in 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. 3*A*). 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. 3*A*). 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	+	+

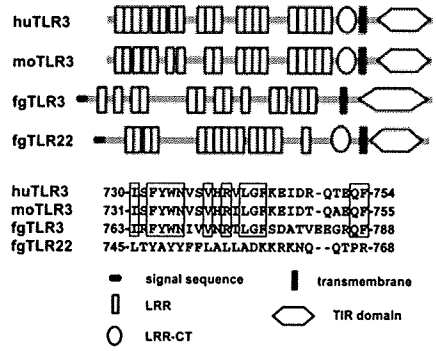


Fig. 3. Different TLRs cover surface dsRNA recognition in fish and mammals. (A) TLR3 and TLR22 in vertebrates. In human and mouse, TLR3 encompasses cell surface and endosomal RNA sensing and induces IRF-3 activation. In fish, two distinct gene products, TLR3 and TLR22, participate in dsRNA sensing. IFN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

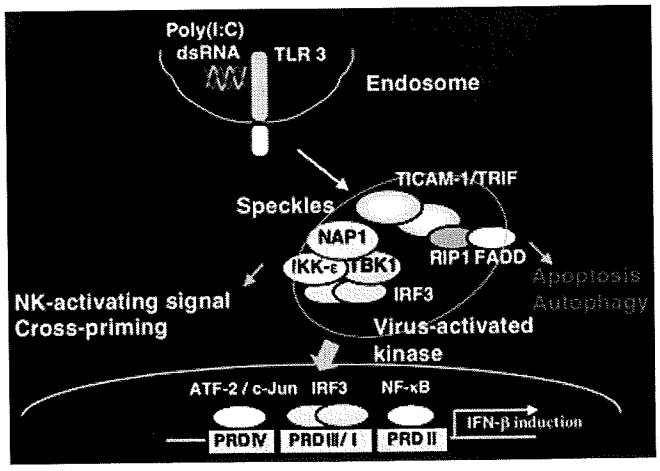


Fig. 4. TICAM-1 is dissociated from TLR3 to form a signaling unit, Speckle. In human cells, TICAM-1 once detached from TLR3 serves as a signaling platform to induce apoptosis, autophagy, NK activation, and cross-priming. TICAM-1 undergoes some modification secondary to complex formation with TLR3 and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

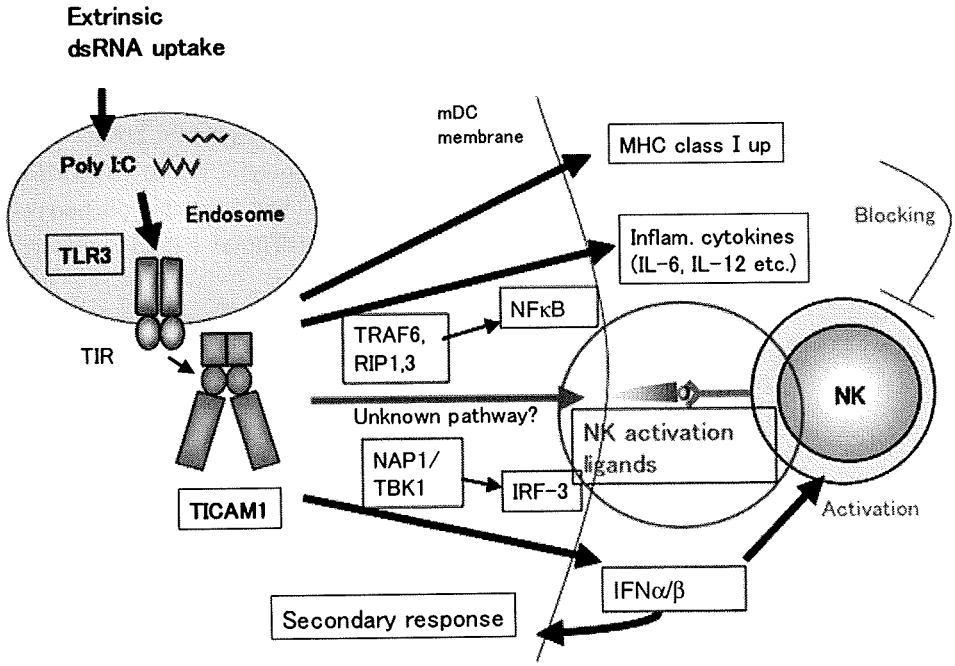


Fig. 5. Mechanism of mDC-NK reciprocal activation induced by dsRNA stimulation of mDCs. TICAM-1 has a crucial role in NK activation driven by polyI:C-stimulated mDCs in human cells. When TLR3 grasps the dsRNA signature in the endosome of mDCs, TICAM-1 in mDCs is activated to evoke a signal pathway reaching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

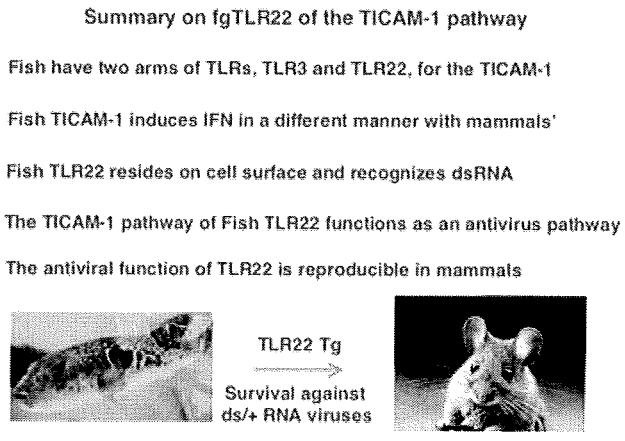


Fig. 6. Summary on fgTLR22 of the TICAM-1 pathway. (i) Fish have two arms of TLRs, TLR3, and TLR22, for the TICAM-1. (ii) Fish TICAM-1 induces IFN in a different manner with mammals. (iii) Fish TLR22 resides on cell surface and recognizes dsRNA. (iv) The TICAM-1 pathway of fish TLR22 functions as an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

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Review Article

Pattern recognition receptors of innate immunity and their application to tumor immunotherapy

Tsukasa Seya,[1] Hiroaki Shime, Takashi Ebihara, Hiroyuki Oshiumi and Misako Matsumoto

Department of Microbiology and Immunology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

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Dendritic cells (DC) begin maturation in response to complex stimuli consisting of antigens and pattern molecules (PAMP) for the activation of the immune system. Immune adjuvant usually contains PAMP. Infection represents one event that is capable of inducing such a complex set of stimuli. Recently, DC were subdivided into a number of subsets with distinct cell-surface markers, with each subset displaying unique differential maturation in response to pattern molecules to induce various types of effector cells. In the present study, we review how pattern recognition molecules and adaptors in each DC subset drive immune effector cells and their effect in the stimulated DC. Although tumor cells harbor tumor-associated antigens, they usually lack PAMP. Hence, we outline the properties of exogenously-added PAMP in the modulation of raising tumor immunity. In addition, we describe the mechanism by which DC-dependent natural killer activation is triggered for the induction of antitumor immunity. (*Cancer Sci* 2010; 101: 313–320)

Adjuvants are typically administered with target antigens in order to enhance the host immune response. Freund complete adjuvant (FCA), Freund incomplete adjuvant (FIA), and hydrated alumina (alum) are representative adjuvants that are used as antigen conjugates to potentiate immune responses and antibody production in animals. Although the mechanism by which these reagents enhance immunity was not completely understood, it appeared that the addition of adjuvants to antigens potentially induced immunity by "making it dirty".[1] However, more recently the agonistic features of adjuvants for pattern-recognition receptors (PRR) have been highlighted based on elucidation of the ligand properties of Toll-like receptors (TLR) and TLR-mediated dendritic cell (DC) maturation. The accumulated evidence on TLR-dependent DC maturation has solidified the current understanding that DC TLR confer the direction of the effector driving on the DC that present antigens. We hold that antigens determine the object toward which immune cells are proliferated, whereas adjuvants determine what effectors will be selected for immunological output.[2] The fundamental concepts of the immune system should be re-evaluated through the understanding of TLR-mediated DC immune responses, which will also revolutionize the concepts related to antitumor immunity.

The two major arms of the innate immune signaling pathway, the MyD88 and toll-interleukin 1 receptor domain (TIR)-containing adoptor molecule (TICAM-1) pathways (Fig. 1), have been identified through the investigation of TLR signaling.[3] Although MyD88 is dominant in mammals living on land, most aquatic vertebrates preferentially use TICAM-1 for TLR signaling.[4] TLR employing MyD88 adaptors usually recognize bacterial patterns, whereas TLR taking TICAM-1 recognize virus products, including nucleic acids. In addition to these PRR, the retinoic acid-inducible protein I (RIG-I)-like receptor and nucleotide-binding oligomerization domain-containing protein (NOD)-like receptor (NLR) systems are located in the cytoplasm[5,6] and are inherent in most animals.[7] PRR systems are also distributed across the cell membrane and cytoplasm. The mineral oil component of FIA, crystallized uric acid, and alum are able to activate the NLR-inflammasome pathway,[5] which yields interleukin (IL)-1β and IL-18. These cytokines in turn stimulate their respective receptors to activate the MyD88 pathway in myeloid DC (mDC).[8] The activation of the MyD88 pathway in mDC is a common feature in bacterial stimulation.

The MyD88 pathway of plasmacytoid DC (pDC) is unique, as TLR7 and TLR9 predominantly activate interferon-regulatory factor (IRF)-7 and induce interferon (IFN)-α.[9] Human mDC lack TLR7 and TLR9 and the IFN-inducing MyD88 pathway, although mouse mDC harbor the TLR7 and TLR9 MyD88 pathway, which are inducible by RNA and CpG DNA respectively.[10] In contrast, TICAM-1 links the type I IFN-inducing pathways in the mDC of both humans and mice,[11] while TLR3 represents the sensor of dsRNA of viral origin.[12] In addition, viral products, double-stranded (ds) RNA, and 5'-triphosphate RNA stimulate the intracytoplasmic helicases melanoma-differentiation-associated gene 5 (MDA5) and RIG-I, which in turn activate the IRF-3- and IRF-7-activating kinases (TANK-binding kinase (TBK1)/I kappa B kinase (IKK)ε.[5,11] The adaptor of this pathway is IPS-1,[5] and it is therefore known as the interferon-beta promoter stimulator 1 (IPS-1) pathway. The IPS-1 pathway shares the downstream signaling components, including the kinases, with the TICAM-1 pathway to activate the IFN-inducing pathway.[13] Thus, the representative inflammatory responses in pattern recognition are rooted in the properties of the adaptors in the case of TLR, MyD88, and TICAM-1. In DC, these pathways play a significant role in differential maturation.

Bacterial and viral pattern molecules revisited

It is known that FCA contains heat-killed mycobacteria (the causative agent of tuberculosis), which functions as a ligand of TLR.[14] These are MyD88-dependent properties and the features of the DC maturation profiles with these TLR ligands have been examined (Table 1). Although the toxicity of the TLR agonists is not removed, their role in triggering antitumor immunity, including cytokine- and effector-inducing abilities, are being examined with respect to their practical use for patients with cancer. Alum (aluminum hydroxide) acts as an NLR agonist involving the secondary activation of MyD88[15] and is currently used as a standard adjuvant in humans. However, a sufficient immune potential may not be accomplished with a

[1]To whom correspondence should be addressed.
E-mail: seya-tu@pop.med.hokudai.ac.jp

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single stimulation of the NLR system. The adjuvant BCG– cell wall skeleton (CWS), which contains mycolic acid, arabinogalactan, and peptidoglycan (PGN), has been used for patients with cancer, and a good prognosis was reported after BCG–CWS treatment.[16] This adjuvant contains muramyl dipeptide (MDP) as a center for the activation of TLR2 and TLR4 and also involves MyD88 activation.[17] The DC maturation profile induced by BCG-CWS is comparable to that induced by Pam2 peptides that activates TLR2 (4) BCG–CWS does not contain DNA, which excludes the possibility of activating TLR9. Only rare examples of fatal shock and interstitial pneumonia have been reported with BCG–CWS that stimulates TLR2 and TLR4.[18]

In contrast, viral products, including dsRNA (and its analog polyI:C), and the lipopolysaccharide (LPS) of Gram-negative bacteria were identified as TLR ligands with TICAM-1 agonistic function.[3] dsRNA and LPS stimulate TLR3 and TLR4, respectively, both of which link the adaptor TICAM-1.[3,11] As they activate nuclear factor (NF)-κB and IRF-3, cytokine storm (hypercytokinemia) or endotoxin-like shock tends to occur *in vivo*.[19] It is therefore mandatory to reduce their toxic properties before they are applied to human patients. Importantly, polyI:CLC (TLR3-complexed poly inosinic: polycytidylic (IC) with carboxymethylcellulose and poly-L-lysine to improve resistance to ribonucleases (i.e. TLR3),[20] and monophosphoryl lipid A (i.e. TLR4)[21] have been considered promising candidates for immunotherapy. These TLR agonists mainly stimulate the TICAM-1 pathway without the robust activation of the MyD88 pathway[20,21] and rarely induce side-effects, such as cytokine storms, skin festering, and the symptoms of inflammation during preclinical trials. It is important that the differential view of the MyD88 and TICAM-1 adjuvants in terms of their DC maturation and effector-driving properties be examined. The development of TLR agonists with properties superior to those of alum can be expected to be revealed through these studies. In this review, the molecular mechanisms of effector activation by DC TLR are outlined and discussed.

Adjuvants stimulate tumor-associated myeloid cells and DC

We have speculated from *in vitro* studies that immature mDC are matured to antigen-presenting mDC by BCG–CWS, a TLR2 agonist,[22] which also induces a variety of immune effector cells, including CD8+ T cells (CTL)[23] and NK cells.[24] These effector cells can damage tumor cells under high effector target (E/T) ratios *in vitro*.[23,24] Indeed, tumor B16 mutanoma growth is retarded in tumor-bearing mice (C57BL/6) when BCG–CWS-matured mDC or secondary-induced CTL are injected in the area surrounding the tumor. It is the CTL, but not NK, cells that are the main effector responsible for tumor regression *in vivo*.[23] Unexpectedly, however, the immune cells which infiltrate into the tumor largely consist of macrophages and not lymphocytes or mDC in mouse models (Shime H and Seya T, unpublished observation, 2009). The properties of these macrophages remain experimentally undetermined. As the tumor-infiltrating macrophages contain many subsets, and some of them often possess immune suppressing properties,[25] these macrophages could be related to myeloid-derived suppressor cells (MDSC) and act as inflammation inducers to sustain tumor growth. Thus, BCG-CWS-mediated functional modification of these macrophages and their effect on tumor growth in mice remains to be determined. Specific questions also remain concerning adjuvant administration to patients. How myeloid cells mature to DC after they are phagocytozing tumor-associated antigens, how mature mDC are located by effector cells, and how tumors regress in such situations still remain unanswered.

Treg, a regulatory population of CD4 T cells, has an inhibitory activity against antitumor immunity[26] and has been shown to inhibit CD8 CTL tumoricidal activity *in vitro*.[26] Several reports indicate that Treg cells infiltrate into tumors and support tumor progression.[27,28] However, mDC are present at very low levels in the tumor masses where Treg cells invade. Again, the functional modulation of Treg cells in the local tumor environment by adjuvants or mDC is unclearly illustrated.

Recently, several myeloid cell populations have been discovered that are associated with tumor cell progression, including interferon-producing killer DC (IKDC),[29] MDSC,[25,30] and tumor-associated macrophages (TAM).[31] Although the maturation or activation of these myeloid cells is likely crucial for tumor progression, only a few reports have investigated their maturation mechanism and effect on tumors by adjuvant treatment. Early-acting pattern molecules can act on tumor cells to release late-acting substances. In fact, damage-associated molecular patterns (DAMP), such as high-modify group box protein (HMGB1), uric acids, heat-shock protein (HSP), and DNA complexes,[32] are secondary liberated from tumors, and stimulate the TAM. Whether these stimuli alter the tumor-progressing ability of the macrophages should be a point of consideration for adjuvant therapy. The types of TLR present in these myeloid cells and the effect of administered adjuvants are topics that need to be investigated.

Many studies on TLR knockout mice allowed us to describe the properties of mouse bone marrow-derived DC (BMDC) treated with a variety of adjuvants[33] and to show the points for induction of immune effector cells through the adjuvant immunotherapy of cancer. Ambivalent functions between mDC and MDSC in a tumor environment can affect the conformation of antitumor immunity.

MyD88- and TICAM-1-mediated DC maturation

Soon after the discovery of the TLR,[34] it was shown that TLR agonists have a DC maturation activity.[35] DC maturation is characterized by TLR adaptors, which have common features, including the upregulation of major histocompatibility complex (MHC), costimulators and NK-activating ligands, and the following features which are unique to each adaptor in mDC.[36] MyD88-dependent DC maturation has two modes, with NK activation and CTL induction occurring concomitantly with the activation of NF-κB, followed by the induction of inflammatory cytokines.[37] Using BCG–CWS as an adjuvant for the TLR2 agonist, we examined how the TLR2 agonist acts on mDC and tumor cells.[23] While NK activation by MyD88 is feasible *in vitro*, TLR2 agonists exhibit minimal NK-mediated tumor-suppression activity in tumor-implant mice.[24] The TLR2-dependent antitumor NK activity is abrogated in MyD88–/– mice, suggesting the presence of a NK-activation pathway via MyD88.[24] However, following an *in vitro* analysis, it was revealed that TLR2–MyD88 in NK cells, but not in mDC, is rather dominant in this mode of NK activation, and that activated NK cells barely enter the tumor mass. For this reason, the subcutaneous administration of BCG–CWS marginally retards tumor growth in mice via the activation of NK cells.

In contrast, mDC maturation is accompanied with potent antigen presentation secondary to cross-priming in TLR2-primed mDC.[23] Tumor antigen-specific CTL induction is facilitated in mice with an implant tumor burden, concomitant with the retardation of tumor growth. This CTL induction is MyD88 dependent, since TLR2-mediated cross-priming does not occur in MyD88–/– mDC. Neither CTL induction nor the retardation of tumor growth significantly occurs in MyD88-deficient mice. Thus, MyD88 in mDC preferentially participates in cross-priming and driving CTL *in vivo*. The downstream molecules of MyD88 associated with mDC CTL driving are unknown.

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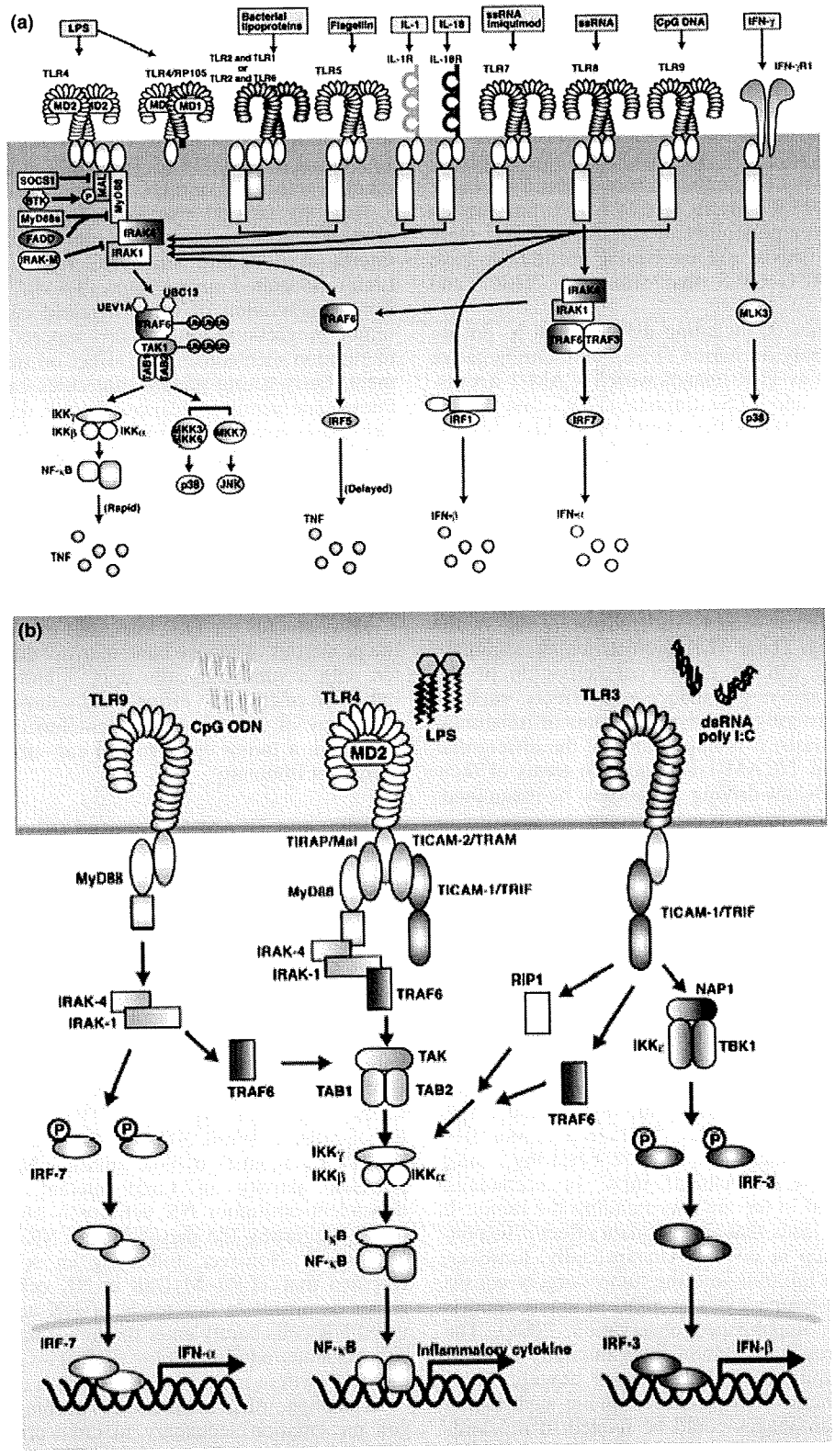

Fig. 1. MyD88 and Toll-interleukin I receptor-domain (TIR)-containing adoptor molecule (TICAM-1) pathways. MyD88 is an adaptor for all Toll-like receptors (TLR), except TLR3 (a). TLR2 and TLR4 recruit MyD88 via the bridging adaptor Toll-interleukin I receptor-domain (TIR)-containing adoptor protein (TIRAP) (MAL) (b). Other TLR directly recruit MyD88. MyD88 activates nuclear factor-kappa β (NF-κB) in most cell types, except plasmacytoid dendritic cells (pDC), which activate the interferan-regulatory factor (IRF-7) transcription factor. MyD88 pathway is involved in the production of pro-inflammatory cytokines in most cells. In contrast, the MyD88 pathway in pDC and the TICAM-1 pathway in myeloid dendritic cells (DC) activate the type interferon (IFN) promoter via IRF-3 or IRF-7 (b). TLR4 can recruit both MyD88 and TICAM-1, whereas other TLR recruit either of them. Each TLR responds to different agonistic stimuli, as shown in Table 1. DC, dendritic cells; IFN, interferon; IRF, interferon-regulatory factor; pDC, plasmacytoid DC; TICAM-1, Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule; TIRAP, Toll-interleukin 1 receptor (TIR) domain-containing adapter protein; TLR, toll like receptor.

Table 1. Human TLR and pattern molecules with MyD88- or Toll-interleukin I receptor domain (TIR)-containing adoptor molecule (TICAM-1)-activating properties

Human TLR	Ligands
TLR1	Pam3
TLR2	Pam2, Pam3, PGN
TLR3	dsRNA
TLR4	LPS, virus fusion units
TLR5	Flagellin
TLR6	Pam2
TLR7	ssRNA
TLR8	ssRNA
TLR9	CpG DNA
TLR10	–

MyD88 activators (Lipoproteins, PGN)	Reference
M161Ag (MALP-2)	(62)
TAN33	(63)
OM-174	(64)
BCG-CWS (Azuma lot)	(22)
SMP105	(65)

TICAM-1 activators (RNA, lipid A)	
DI RNA (stem loop)	(66)
Poly(A:U)	(67)
Poly(I:C_{12}U)	(68)
PolyI:C(LC)	(20)
MPLA	(21)

Anti-human TLR monoclonal antibodies		
TLR1	TLR1.136	(58)
TLR2	TLR2.45	(59)
TLR3	TLR3.7	(60)
TLR4	HTA125	(61)
TLR6	TLR6.127	(58)

dsRNA, double-stranded RNA; TLR, Toll-like receptor. SMP105 is a lot of BCG-CWS that activates only TLR2.

In the present study, we used polyI:C for evaluating the TICAM-1 potential in mDC maturation and antitumor immunity.[38] The TICAM-1 pathway allows mDC to activate IRF-1 and IRF-3, which in turn activate the IFN-β promoter, as well as unidentified antitumor factors (Fig. 1). The data imply that cross-priming and the NK-driving signal are also dependent upon TICAM-1, but the transcription factors utilized by TICAM-1 are wholly distinct from those of MyD88. The search for the molecules that participate in the TICAM-1 CTL driving is underway, and a molecule downstream of IRF-1, but not IRF-3, has been shown to be crucial for *in vivo* CTL induction. In contrast, TICAM-1-mediated antitumor NK activation largely relies on the IRF-3-derived NK-activating molecule (INAM), in addition to the reported cytokines IL-15, IFN-α, and IL-12p70.[39]

MyD88 and TICAM-1 activate different signaling platforms for the recruitment of second adaptors.[3] In mDC, TLR2 and TLR4 recruit the combined adaptor Mal/Toll-interleukin I receptor domain-containing adoptor protein (TIRAP)–MyD88 to signal the transcription factor NF-κB.[4] In contrast, TLR3 and TLR4 can utilize TICAM-1 as the adaptor.[3] TLR4 recruits the combined adaptor Toll-IL-IR domain-containing adoptor inducing IFN-beta-related adaptor molecule (TRAM) (TICAM-2)–TICAM-1 while TLR3 directly recruits TICAM-1 for signaling.[3] TLR4 is unique in that it uses both MyD88 and TICAM-1 adaptors (Fig. 1). The classic example in which both routes are activated is during LPS-induced endotoxic shock.[40] Like BCG-CWS and PolyI:C, activation of either one route would be required for a condition of less toxic adjurants. Studies of the TICAM-1 signalosome suggest that upon TLR3 activation, TICAM-1 recruits a variety of molecules as secondary adaptors, including NAK-associated protein I (NAP1),[41] receptor-interacting protein I (RIP1),[42] similar to NAP I TBK adoptor (SINTBAD),[43] adenovirus 5 E1A-binding protein (BS69),[44] and TNF receptor-associated factor (TRAF) family proteins.[45] Whether or not these molecules are associated with antitumor CTL or NK induction remains to be determined.

The mode by which mDC are matured differs in the MyD88 and the TICAM-1 pathways. The TICAM-1 pathway preferentially induces IL-12 and type I IFN in mDC and drives NK activation.[38] Type I IFN induction by MyD88 has been observed only in pDC.[9,10] In contrast, mDC MyD88 strongly induces pro-inflammatory cytokines, such as tumor necrosis factor-α, IL-1β, and IL-6.[9,46] The molecular mechanism that facilitates the cross-presentation ability in mDC is currently unknown.

DC subsets and TLR expression

BMDC (representative of mDC) and pDC can be prepared from mouse bone marrow cells by using granulocyte-macrophage colony-stimulating factor (GM–CSF) and the Flt3 ligand[46] while Langerhans cells can be generated by the addition of transforming growth factor-β to GM–CSF and IL-4.[47] The DC subsets in the spleen and the intestinal tract can be separated using a flow cytometry (FACS) sorter. The characteristics of these mouse DC subsets have been described previously.[36] In humans, monocyte-derived DC can be used as mDC, but their characteristics are somewhat different from mDC prepared in the peripheral blood using the mDC marker plasmocytoid DC antigen (PDCA1). Human peripheral blood pDC can be isolated from whole blood using PDCA4.

The distribution of TLR of the DC subset were examined by using human TLR-specific monoclonal antibodies generated in our laboratory, and the TLR repertoires of monocyte-derived DC and pDC were determined (Table 2). The TLR distribution roughly resembles mouse DC, although a clear result could not be obtained with mouse BMDC and pDC because of a lack of appropriate specific antibodies against mouse TLR.[36] The discrepancy of appropriate TLR7 levels in mouse BMDC and human mDC could be a result of differences in the inducible nature of mouse, but not human, TLR7. It was also shown that human mDC express TLR8, while mouse mDC do not.[36] The

Table 2. TLR expression profiles in human DC subsets

	Freshly isolated			*In vitro*-differentiated	
	Monocytes	mDC*	pDC**	DCs	Macrophages
TLR1	++	+	–	+	++
TLR2	++	++	–	++	++
TLR3	–	++	–	++	+
TLR4	++	+	–	+	+
TLR6	++	+	–	+	+
TLR7	–	–	+	–	–
TLR8	+	+	–	+	+
TLR9	–	–	+	–	–

Positive and negative symbols denote the results of the flow cytometry (FACS) analyses using monoclonal antibodies, except TLR7, TLF8, and TLR9. Results were determined by reverse transcription–polymerase chain reaction. TLR3, TLR7, TLR8, and TLR9 reside in the endosome to recognize nucleotide derivatives. (*) PDCA1+ cells; (**) PDCA4+ cells. PDCA, plasmacytoid dendritic cell antigen; DC, dendritic cell; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; TLR, Toll-like receptor.

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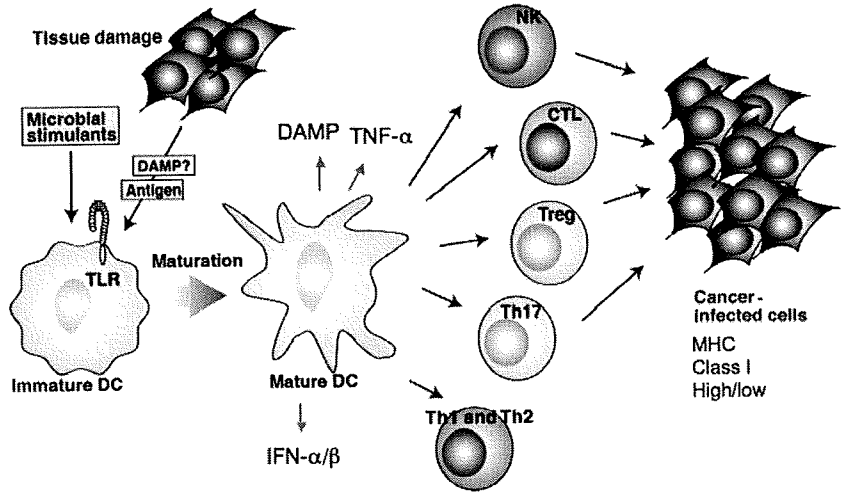

Fig. 2. Selective induction of immune effector lymphocytes by different agonistic stimuli. Each pattern molecule (PAMP) has its own uniqueness in myeloid dendritic cell (mDC) maturation. Differential maturation of mDC results in different effector driving as shown. CD8 T, various CD4 T, and B cells are proliferated by myeloid DC (mDC) with different properties. Tumor regression is a marker for evaluating which lymphocytes are activated in response to pathogen-associated molecular patterns (PAMP).

failure of CpG DNA to raise effective antitumor immunity can be attributable to the low or absent induction of TLR9 in human mDC, unlike the situation in mouse mDC.

DC subsets and effector induction

CTL and NK cells can be induced by mature mDC, with or without the presentation of MHC class I antigens, while CD4 T-cell subsets are induced by the presentation of class II antigens. In addition to CTL and NK cells, the tumor-modulating functions of Th1, Th2, Th17, and Treg were evaluated (Fig. 2). NK activation is a result of the balance between NK-activating and inhibitory ligands on mDC. NK cells can also be activated with cytokines, such as IL-2, IL-15, IFN-α/β, and IL-12.[48] CTL is a result of the activation of the CD8+ T cell by the presentation of class I antigens on mDC. Other effectors are the result of the activation of CD4+ T cells by MHC class II antigen presentation on mDC. A master transcription factor in addition to T-bet, GATA-3, RORgT, and Foxp3 are known to exist for Th1, Th2, Th17, and each Treg on the CD4 lymphocyte side.[49] However, there is little information concerning the mDC properties driving these effector cells.

Each DC subset seems to correspond to a specific effector, although the selection mechanism by which DC induce various effectors is not clear in most instances. However, it is known that CD8+ DC induce Treg[50] and NK cells[51] in the mouse spleen, and lamina propria pDC in the mouse enteric canal promotes immunoglobulin A production.[52] In addition, CD70+/CD11c+ DC induce Th17 cells by the adenosine tri phosphate (ATP) of enterobacteria,[53] and BMDC activate NK cells via the TICAM-1 pathway.[54] Further examples of DC subsets that preferentially function with specific effectors will likely be demonstrated through practical experiments.

Mechanism of DC-mediated antitumor NK activation

It has been reported that BMDC drive antitumor NK activation in a TICAM-1-dependent manner.[38,54] This NK activation does not rely upon a soluble factor, such as a cytokine, but instead was generated by BMDC–NK cell–cell contact.[39] Therefore, there must be an NK-activating molecule that is induced on the BMDC surface in response to TICAM-1 signaling (Fig. 3a). We focused our attention on this key molecule, which is crucial for antitumor NK immunity and found that DC-mediated NK activation occurred normally in IRF-7 −/− BMDC stimulated with polyI:C, but this response was absent in IRF-3 −/− BMDC.[39] Therefore, the putative NK-driving signal in mDC involves transcription factor IRF-3 downstream of the activated TICAM-1. Ultimately, the NK activation molecule was identified using a screening method in which candidate molecules were expressed in IRF-3 −/− BMDC using a lentiviral vector.[39] We named this molecule

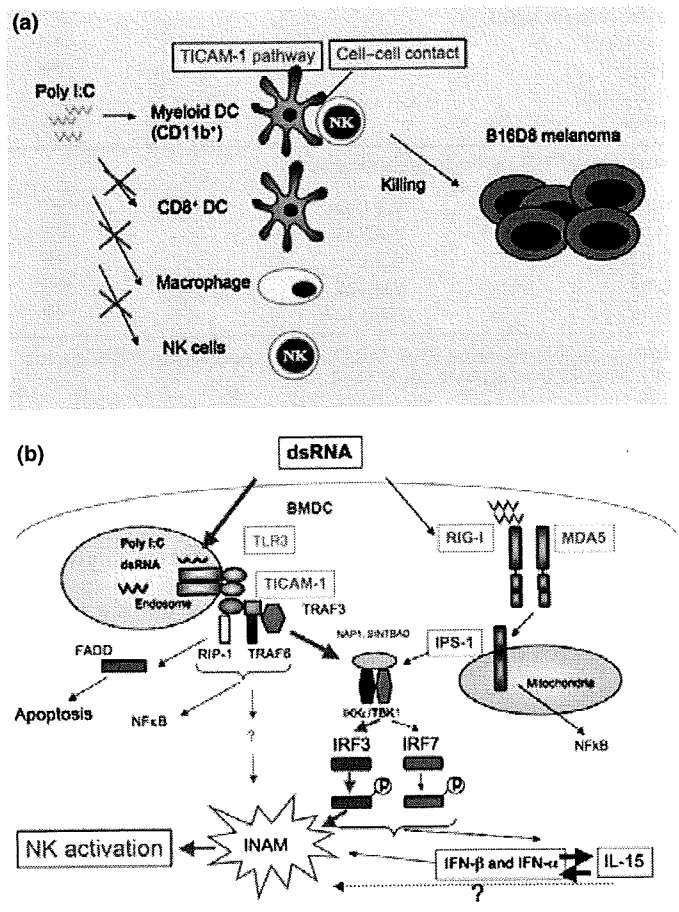

Fig. 3. A molecular mechanism of myeloid dendritic cell (mDC)-mediated natural killer (NK) activation. (a) CD11b+ bone marrow-derived dendritic cells (BMDC) act for natural killer (NK) activation by double-stranded (ds) RNA. NK cells express tumoricidal activity against major histocompatibility complex (MHC) low implant tumors if they are primed by polyI:C plus bone marrow-derived dendritic cells (BMDC), but not other myeloid cells. Dendritic cell–NK cell–cell contact is essential for the induction of polyI:C-mediated antitumor NK cells. (b) Route for mDC maturation for the induction of NK activation.[39]

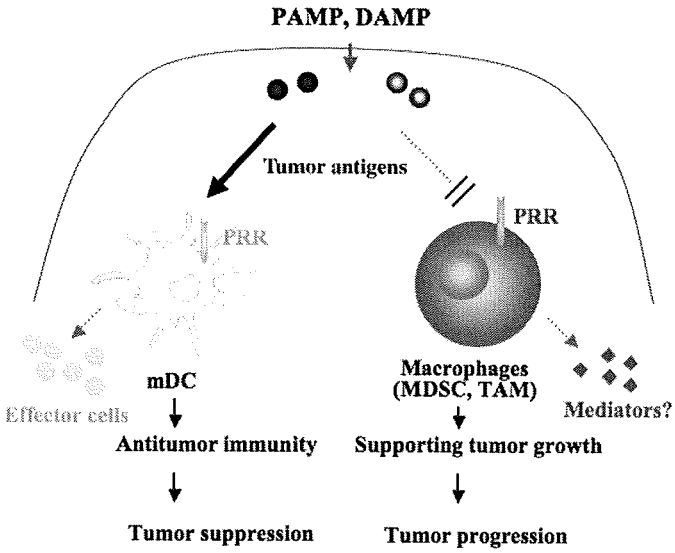

Fig. 4. Diverged functions of myeloid cells in tumor mass. A variety of myeloid subsets reside in tumor masses. Some of the subsets exhibit an immune suppressive feature that facilitates escape of tumor cells from immune effectors. Since pattern molecules (PAMP) act on both myeloid dendritic cells (mDC) and myeloid-derived immune suppressing cells, complicated immune responses occur in tumors. Selective maturation of mDC circumventing the exacerbation of tumor progression by myeloid suppressor cells should be considered as adjuvant therapy of cancer. DAMP, damage-associated molecular patterns; MDSC, myeloid-derived suppressor cells; PRR, pattern-recognition receptors; TAM, tumor-associated macrophages.

INAM. When INAM was expressed in mDC, it promoted NK activation in the mixture of mDC (expressing INAM) and NK cells; however, INAM did not exhibit an NK-activating function on BaF3 cells. INAM is an NK-activating molecule peculiar to BMDC whose TICAM-1 has been activated, and there have been no reports suggesting the presence of this kind of molecule until recently (Fig. 3b). In BMDC, INAM receives a sugar chain modification by a similar membrane protein to tetraspanin with a molecular weight of 45 kDa. INAM is distributed in the spleen and lymph nodes, and is actually expressed by a variety of lymphocyte subsets present in the lymph nodes. It has been predicted to make a loop card structure on the surface of the cell in two portions based on the amino acid sequence.[39]

It is predicted that INAM is related to the composition of immune synapses in the BMDC–NK contact. When BMDC, which forcibly express INAM, are prepared and adoptively transferred around the tumors of tumor-bearing mice, the tumor is efficiently regressed. These results suggest that INAM is the factor directly responsible for driving antitumor NK activation. Humans have an ortholog of INAM, although its distribution profile appears to be somewhat different than to that of mice.

Points to trigger antitumor immune potential

Effector tumor cell–cell contact is essential for tumor damage by immune effector cells. The material liberated from cancer cells on one side generates the modulators of the PRR of mDC and influences the trigger of effector induction. The host molecules that modulate PRR are the previously-mentioned DAMP.[32] For effective tumor damage, the effector must reach the tumor mass. A suitable strategy is needed for determining the basic factor(s) of the immune response involved in cancer, and can be achieved by using immunomodulatory reagents and gene-disrupted mice with abrogated TLR pathways.

We have analyzed how BMDC acquire effector-driving functions by focusing on the innate immune response. The results suggest that PRR stimuli become a trigger that leads to the alteration of precancerous cells to the malignant form. However, PRR are indispensable to the activation of antitumor immunity. In both cases, myeloid cells are intimately involved in the process of tumor–immune cell interaction. Indeed, BCG has high therapeutic potential for patients with bladder transitional epithelial cancer,[55] but it has less of an effect on a variety of other solid cancers. This discrepancy can be rooted in the fact that myeloid cells interact with tumor cells with ambivalent reaction profiles (Fig. 4). An effective strategy for tackling the issue of immune abnormality has yet to be proposed, and even the fundamental immune aberrance present in the microenvironment of tumors is not generally recognized by researchers. It has been speculated that tumor cells produce cytokines that modulate the inflammatory environment as tumor develops. When tumor is surgically excised, many constitutional accidents are often diminished,[56] which can reflect the fact that tumors develop concomitantly with immune modulation. It has become clear that some modulating factors of the innate immune system, such as DAMP, cause cancer-mediated idiosyncrasies (Fig. 2).

Up until now, the effectiveness of cancer immunotherapy has been primarily evaluated based on tumor regression and the survival prognosis of patients. A representative study involved the evaluation of peptide vaccine therapy for cancer treatment. According to the report by Rosenberg,[57] the peptide vaccine administered to melanoma patients had an effective rating of approximately 2.6%. For future studies, it is necessary to determine the potential of peptide-conjugating materials, including adjuvants and inflammation-inducing reagents.[20] A number of reports have suggested that adjuvants can greatly increase the efficiency rate of treatment, although the criteria is prerequistie to fairly evaluate the function of adjuvants in cancer patients.

The method for stimulating DC needs to be carefully selected, as the systemic administration of inflammation-inducing material can also lead to the acceleration or invasion of developing malignancies at the same time (Fig. 4). The adoptive transfer of adjuvant-treated mDC to patients is a promising choice; however, it might be difficult for this treatment to be adapted by the Japanese health insurance system. The molecular manipulation of a specific PRR in DC that is involved in effector driving can lead to effective treatment with minimal side-effects. In this case, the route and molecule that selectively raises the degree of DC maturation without enhancing MDSC should be clarified. If the inflammatory signals that promote carcinogenesis are properly controlled using adjuvants, the design of DC maturation can be manipulated without helping tumor progression. The search for the functional molecule of antitumor effector induction in mDC will help establish an effective treatment of cancer and facilitate the evaluation of the efficacy of peptide vaccines. In the future, we hope that through continued research, cancer patients will have access to convenient and highly effective immunotherapy.

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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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Hiroyuki Oshiumi[‡], Misako Matsumoto[‡], Shigetsugu Hatakeyama[§], and Tsukasa Seya[‡1]

From the [‡]Department of Microbiology and Immunology and the [§]Department of Biochemistry, Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Kita-ku Sapporo 060-8638, Japan

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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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB470605.

[1] To whom correspondence should be addressed: Dept. of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo 060-8638, Japan. Tel.: 81-11-706-5073; Fax: 81-11-706-7866; E-mail: seya-tu@pop.med.hokudai.ac.jp.

[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-

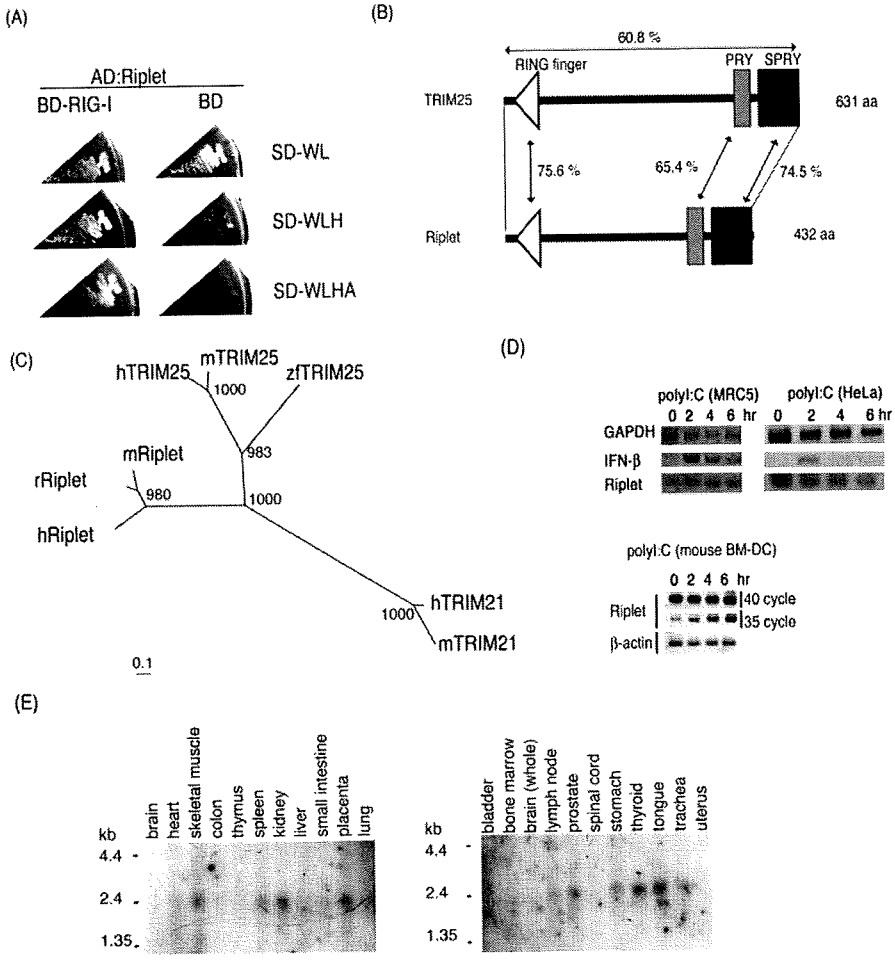

FIGURE 1. **Isolation of Riplet by yeast two-hybrid screening.** *A*, yeast cells carrying both RIG-I and Riplet can grow in selective media (SD-WLH, SD-WLHA), whereas yeast cells carrying RIG-I alone only grow in nonselective media (SD-WL), indicating the physical interaction of RIG-I with Riplet. *B*, human Riplet protein sequence is 60.8% identical to human TRIM25. The RING finger domains and SPRY motifs show higher sequence similarities between the two proteins. *aa*, amino acids. *C*, phylogenetic tree constructed by the Neighbor-Joining method shows that Riplet is similar to TRIM25. *h*, *m*, *r*, or *zf* represent human, mouse, rat, or zebrafish, respectively. The *numbers* on the node are bootstrap probabilities ($n = 1000$). *D*, HeLa cell, human primary-cultured fibroblast cell, MRC5, or bone marrow-derived mouse dendritic cell (*BM-DC*) were stimulated with poly(I-C) (50 μg/ml) for indicated hours. Total RNA was extracted with TRIzol reagent, and then RT-PCR was carried out using primers shown under "Experimental Procedures." *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *E*, Northern blot membranes containing 1 μg of poly(A)$^+$ RNA per lane from human tissues were blotted with human Riplet probe.

tor was added to normalize the final plasmid amount. 48 h after transfection, cells were stimulated with poly(I-C) for 4 h. For VSV infection, 24 h after transfection, cells were infected with VSV at m.o.i. = 1, and cell lysate was prepared after 12 h for reporter gene assays. The degree of gene silencing was confirmed by RT-PCR using RNA extracted from cells 24 h after transfection. PCR primers used for the RT-PCR were Riplet-F3 (ACTGGGAAGTGGACACTAGG) and Riplet-R3 (ACTCATACAGAAGCTTCTCC). siRNAs were purchased from Funakoshi Co., Ltd. (Tokyo Japan), and the siRNA sequences of Riplet siRNA were GACUAUGGACUCUUGUUGUGU (sense) and ACAACAAGAGUCCAUAGUCCU (antisense). Control siRNA sequences were CUGUUGGUUUAGUAAGCCUGU (sense) and AGGCUUACUAAACCAACAGUC (antisense). Another siRNA, Riplet si-1, and control negative siRNA (silencer negative control 1 siRNA, AM4611) were purchased from Applied Biosystems. siRNA sequences were Riplet si-1 GGGAAAGCUUGCCUUCUAUdTdT (sense) and AUAGAAGGCAAGCUUUCCCdTdC (antisense).

Virus Preparation and Infection—VSV Indiana strain and poliovirus were amplified using Vero cells. HEK293 cells were transfected in 24-well plates with plasmid encoding *RIG-I*, Riplet, or no insert. 24 h after transfection, cells were infected with viruses for 24 h, and the titers of virus in culture supernatant were measured by plaque assay using Vero cells. For RNA interference assay, cells were transfected with siRNA with Lipofectamine 2000. 24 h after transfection, cells were infected with viruses at m.o.i. = 0.001 for 18 h, and the titer in culture supernatant were determined by plaque assay.

Immunoprecipitation—HEK293FT cells were transfected in 6-well plates with plasmids encoding FLAG-tagged RIG-I and/or HA-tagged Riplet. The plasmid amounts were normalized by the addition of empty plasmid. 24 h after transfection, cells were lysed with lysis buffer (20 mM Tris-HCl (pH 7.5), 125 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 30 mM NaF, 5 mM Na_3VO_4, 20 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride), and then proteins were immunoprecipitated with rabbit anti-HA polyclonal (Sigma) or anti-FLAG M2 monoclonal antibody (Sigma). The precipitated samples were analyzed by SDS-PAGE and stained with anti-HA (HA1.1) (Covance) or anti-FLAG M2 monoclonal antibody. For ubiquitination assay of RIG-I, the plasmid encoding two multiple HA-tagged ubiquitins was used. HEK293FT cells were transfected with plasmids encoding FLAG-tagged RIG-I, Riplet, or 2× HA-tagged ubiquitin. 24 h after transfection, cells were lysed, and then RIG-I was immunoprecipitated as described above. The samples were analyzed by SDS-PAGE and stained with anti-HA polyclonal antibody (for detection of ubiquitination) or anti-FLAG monoclonal antibody (for detection of RIG-I). Reproducibility was confirmed with additional experiments (see supplemental figures).

Construction of RIG-I 3KA and 5KA Mutant Genes—The C-terminal three or five lysine residues were mutated into alanines (designated as 3KA and 5KA). RIG-I 3KA has K888A, K907A, and K909A, whereas RIG-I 5KA has K849A, K851A,

K888A, K907A, and K909A. The mutant *rig-I* genes were made by PCR-mediated site-directed mutagenesis. The primers used for the PCR were as follows: K907–909A-forward, GTT CAG ACA CTG TAC TCG GCG TGG GCG GAC TTT CAT TTT GAG AAG, and K907–909A-reverse, CTT CTC AAA ATG AAA GTC CGC CCA CGC CGA GTA CAG TGT CTG AAC; K888A-forward, GAC ATT TGA GAT TCC AGT TAT AGC AAT TGA AAG TTT TGT GGT GGA GG, and K888A-reverse, CCT CCA CCA CAA AAC TTT CAA TTG CTA TAA CTG GAA TCT CAA ATG TC; K849–851A-forward, GAG TAG ACC ACA TCC CGC CCA GCG CAG TTT TCA AGT TTT G, and K849–851A-reverse, CAA AAC TTG AAA ACT GCG CTG GCG CGG GAT GTG GTC TAC TC. PCR was carried with Pyrobest *Taq* polymerase, and the obtained clones were sequenced to exclude the clones harboring PCR error. To construct the plasmid-expressing mutant RIG-I protein, the wild-type *RIG-I* gene on pEF-BOS vector was replaced with the mutant *rig-I* gene.

Real Time PCR—Quantitative PCR analyses were carried out using iCycler iQ real time detection system with Platinum SYBR Green qPCR SuperMix-UDG reagent (Invitrogen). Primer sequences for qPCR were as follows: hGAPDH-qF, GAG TCA ACG GAT TTG GTC GT, and hGAPDH-qR, TTG ATT TTG GAG GGA TCT CG; hIFN-β-qF, TGG GAG GAT TCT GCA TTA CC, and hIFN-β-qR, CAG CAT CTG CTG GTT GAA GA; hMx1-qF, ACC ACA GAG GCT CTC AGC AT, and hMx1-qR, CTC AGC TGG TCC TGG ATC TC; and hIFIT-1-qF, GCA GCC AAG TTT TAC CGA AG, and hIFIT-1-qR, CAC CTC AAA TGT GGG CTT TT. Values were expressed as mean relative stimulations, and for a representative experiment from a minimum of three separate experiments, each was performed in triplicate.

RESULTS

RIG-I-binding Proteins—To isolate the proteins that bind to RIG-I, we performed yeast two-hybrid screening using a human lung cDNA library. Using the RIG-I central region (213–601 amino acids), we isolated a clone that encoded a partial ORF of a gene expressed in a dendritic cell line, DC12, whereas the C-terminal region of RIG-I (557–925 amino acids) resulted in the isolation of two cDNA clones, which encoded partial C-terminal regions of ZNF598 and RNF135 (Fig. 1*A* and data not shown). Preliminary expression studies showed that the RNF135 segment affected the RIG-I IFN-β inducing activity, whereas the other two proteins had no effect (data not shown). We confirmed the