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 TLR22 is ubiquitously expressed in all the organs tested in the Tg mice (A. Matsuo, H. Oshiumi, T. Seya, unpublished data).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performed on Tg mice using influenza virus and poliovirus in an *in vivo* mouse model (A. Matsuo, H. Oshiumi, T. Seya, unpublished data).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

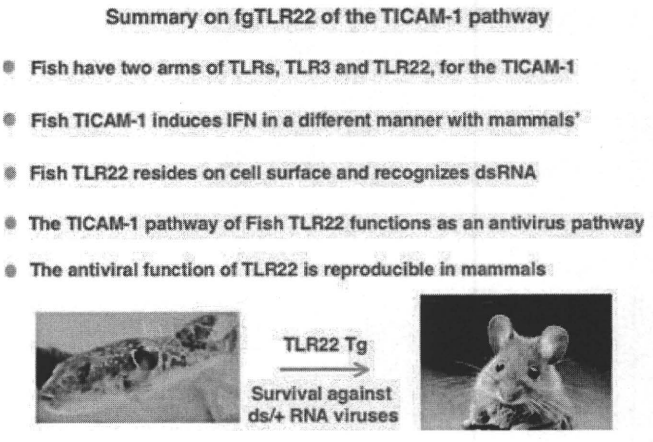

Fig. 6. Summary on fgTLR22 of the TICAM-1 pathway. (i) Fish have two arms of TLRs, TLR3, and TLR22, for the TICAM-1. (ii) Fish TICAM-1 induces IFN in a different manner with mammals. (iii) Fish TLR22 resides on cell surface and recognizes dsRNA. (iv) The TICAM-1 pathway of fish TLR22 functions as an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

The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer

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Abstract Infection with RNA viruses presents a typical pattern of virus products, double-stranded RNA (dsRNA), and induces the maturation of antigen-presenting dendritic cell (mDC). There are several dsRNA sensors that are differentially distributed on the cell membrane and in the cytoplasm and are variably expressed depending on the cell type. Among these sensors, TLR3 links to the adaptor TICAM-1 (TRIF), which is characterized by its unique multipronged signaling cascades for cytokine/chemokine production, apoptosis and autophagy in both immune and tumor cells. In the context of mDC maturation, various cellular events are further induced in response to dsRNA; these include cross-priming followed by CD8+ CTL induction, NK activation and proliferation of CD4+ T cells including Th1, Th2, Treg and Th17 cells. In this review, we focus on the potential role of dsRNA in modulating the inflammatory milieu around mDCs and tumor-associated antigens to drive specific cellular effectors against the tumor.

Keywords Immunotherapy for cancer · RNA adjuvant · Toll-like receptor · TICAM-1 (TRIF) · Dendritic cells · Cellular effectors

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Introduction

Tumor progression often occurs during inflammation because cell growth is an event that is closely connected to both extrinsic and intrinsic inflammatory stimulation [1]. Many biological mediators such as cytokines and chemokines are involved in immune cell recruitment, which accelerates tumor development in an inflammatory milieu [2]. Immune-related cells are incorporated into the tumor matrix and evoke complicated immune responses against the tumor through cell–cell interactions. Ultimately, the antigen (Ag)-presenting cells (APC) mature as a result of the inflammatory stimuli and tumor-associated antigens (TAAs) and flow out to the regional lymph nodes where TAAs are presented to lymphocytes [3]. However, tumor remission does not occur frequently despite TAA presentation by APC [3, 4]. In contrast, most other infections facilitate myeloid dendritic cell (mDC) maturation [5] and provoke a robust immune response that contributes to pathogen eradication. If PRRs fail to be activated due to the lack of appropriate microbial patterns in APC of cancer patients even in the presence of TAAs, no effectors are generated for tumor targeting, thereby neither immune edition nor surveillance occurring against tumor.

Double-stranded (ds) RNA is a product of virus replication. A variety of RNA and DNA viruses generate replication-mediated dsRNA, polyU/UC or stem-loop structures [6], which serve as ligands for pattern-recognition receptors (PRRs). TLR3 [7], TLR22 [8], RIG-I/MDA5 [9], PKR [10], NALP3 [11, 12] and Dicer in the RNAi system [13] along with as yet unidentified receptors are believed to serve as PRRs for dsRNA sensing (Fig. 1a). These PRRs induce intracellular signaling cascades that regulate cell growth, differentiation, apoptosis and immune activation [6, 14]. Ultimately, dsRNA and its synthetic analog polyI:C

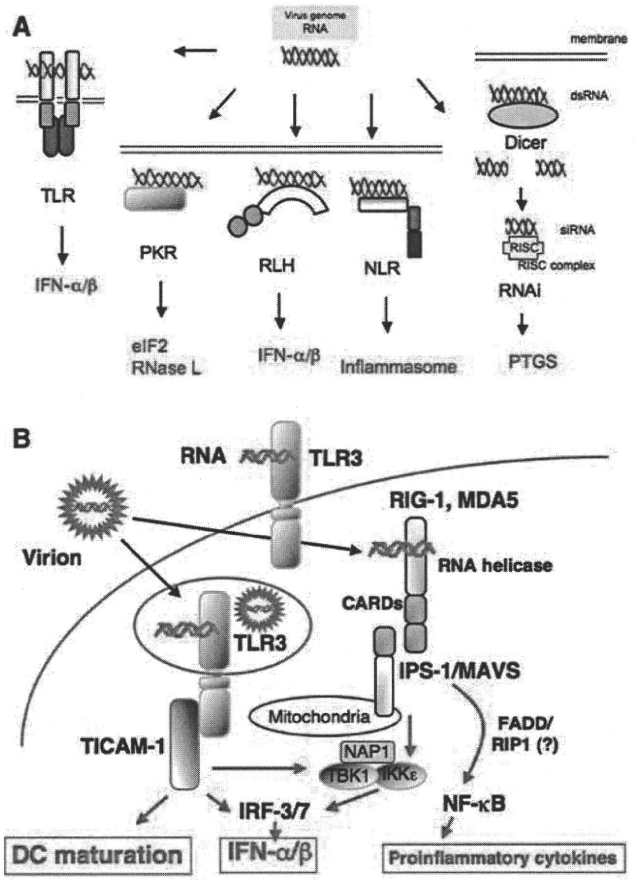

Fig. 1 dsRNA-sensing systems. **a** Double-stranded (ds)RNA are primarily generated during virus replication. Major dsRNA sensors in human cells are indicated. Dicer and RNA-recognizing helicases work in invertebrates as antiviral receptors, but in humans no evidence of these receptors for antiviral action has been proposed. How dsRNA selects a variety of RNA pattern sensors remains largely unknown. PTGS, post-transcriptional gene silencing. **b** TLR3 is mainly localized in the endosome of limited cell types, while RLH (RIG-I and MDA5) are ubiquitously distributed in the cytoplasm. Adaptor 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*

exert a wide range of biological activities and can elicit immune responses. Since dsRNA-sensing PRRs are distributed across a variety of host cells in different combinations [6, 15], systemic inflammation occurs in various modes depending on the receptors and cell types involved in viral infection, virus vaccine inoculation or dsRNA administration for RNA therapy. An inflammatory environment promotes tumor growth and priming of dendritic cells. Many sterile and infectious RNAs induce inflammation.

The signaling pathways of PRRs are linked via adaptor proteins (Fig. 1b). The intra-cytoplasmic RNA sensors, RIG-I and MDA5, interact with MAVS (Cardif/IPS-1/VISA) on the outer membrane of mitochondria [16], and TLR3 resides in the endosome and interacts with TICAM-1 (TRIF) [17]. The signal selection systems of other dsRNA sensors are relatively less defined. Typically, stimulation of the TICAM-1 and MAVS pathways induces type I interferons (IFN) [18]. This is a reflection of the fact that the signaling cascades of both pathways converge upon the complex of the virus-activated kinase (VAK), i.e., NAP1/SINTBAD-IKKe/TBK1 [18, 19] (Fig. 2a). Other cellular responses, autophagy [20], proliferation [21] and apoptosis [22], are induced in cells stimulated with dsRNA (Fig. 2a). Study of the molecular mechanism of these responses is currently underway.

In mDCs, a variety of cellular effectors are driven in response to dsRNA. CD4 Th1, CD8 CTL, NK cells, regulatory T cells (Treg), and Th17 cells are activated/proliferated through dsRNA-stimulated mDCs [15]. Some inflammatory cytokines and chemokines, as well as IFN-inducible gene products are also up-regulated in mDCs. These effectors appear to be independently induced in a situation-dependent manner. However, the molecular mechanisms whereby these variable effectors are differentially induced by mDCs are unknown. We have determined that the TICAM-1 pathway in mDCs is involved in inducing all these effector cell types (Fig. 2b).

In this review, we focus on the TICAM-1 pathway in which cellular effectors are induced by mDCs. We also discuss the involvement of the TICAM-1 pathway in cancer progression and the therapeutic potential of TICAM-1 in antitumor immunotherapy.

TLR3 agonists in cancer immunotherapy

PolyI:C is a representative agonist for human and mouse TLR3 [23]. This compound is believed to be an analog of viral double-stranded RNA (dsRNA) and is a strong inducer of type I IFN in both humans and mice [24]. Initially, polyI:C was regarded as a PKR activator [25]. Later, it was determined that this compound is not only a TLR3 agonist, but also a stimulator of the cytoplasmic RNA sensor, MDA5 [26]. PolyI:C also activates RIG-I [26], but other viral RNA patterns, 5′-triphosphate [27, 28] and polyU/UC [29] may be natural ligands for RIG-I. Earlier, it was reported that polyI:C, which is capable of activating various PRRs, causes endotoxin-like cytokine stormsl; therefore, this compound was deemed to be too toxic for application in clinical therapy [30].

mDCs mature into APCs that drive cellular effectors (Fig. 3). TLR3 resides in the endosome of mDCs [17], senses dsRNA in the endosome, and relays signals to the TICAM-1 pathway, thereby leading to maturation of mDCs [6]. Thus, endosomal stimulation of TLR3 by ligands links to activation of mDCs (Fig. 1b). Certain dsRNA derivatives preferentially activate TLR3 rather than RLH receptors

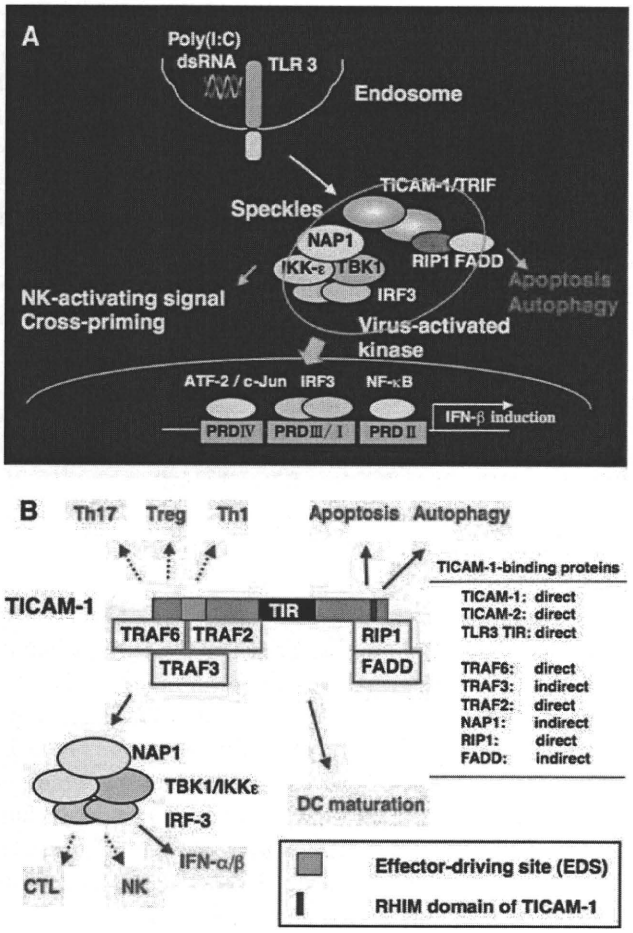

Fig. 2 An outline of the TICAM-1 pathway. **a** In human cells, TICAM-1 once detached from TLR3 serves as a signaling platform to induce apoptosis, autophagy, NK activation and cross-priming. TICAM-1 undergoes some modification secondary to complex formation with TLR3, forms multimer, and dissociated from TLR3 with unknown mechanism. The pathways for NK activation, CTL induction and autophagy are only partially identified, although the pathway for apoptosis is getting clarified. 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 'Effector-driving site (EDS)' recruits appropriate signal-transmitting molecules and matures mDCs leading to induction of effector 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

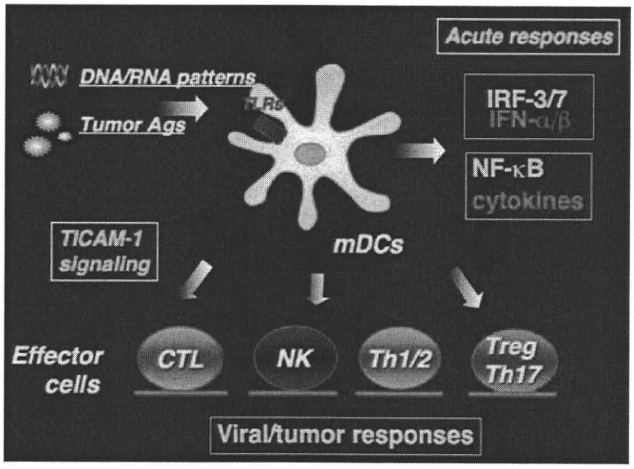

Fig. 3 Various effectors driven by the TICAM-1 pathway in mDCs. The effectors can be induced through the TICAM-1 pathway in mDCs are delineated in this figure. In an early phase of infection, cytokines and IFNs are released in response to microbial patterns. Later, the cellular effectors are induced 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 effect on mice. These modified 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-β 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 identified 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 Effector-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-α/β, proinflammatory cytokines, ROS and K+ are induced in mDCs. Autophagy and apoptosis are evoked in cells other than mDCs. TICAM-1 modification and translocation lead to the formation of TICAM-1 homo-multimers in mDCs, which activate signal pathways leading to induction of cellular effectors, CTL, NK and CD4+ T cells [15]. The IFN-α/β-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-1β, IL-6, TNF-α 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

the inflammasome which may interfere with autophagy. This leads to incremental production of IL-1β 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 different effectors.

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), IKKε 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-α/β 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 IKKα/β and NK-κB [35]. These pathways sustain the production of inflammatory 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]. PolyI:C is a compound that induces autophagy in tumor cells, and this reaction augments the activation of caspase 1 of the inflammasome that produces robust amounts of active IL-1β, IL-18 and IL-33 [49]. TICAM-1 KO cells lose the ability to undergo polyI:C-mediated inflammasome 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 polyI:C [50]. Intestinal epithelial cells of mice are injured upon intraperitoneal administration of polyI: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 polyI: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-1β, TNF-α and IL-8 in response to polyI: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 efficiently 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 efficiently induce Ag-specific 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

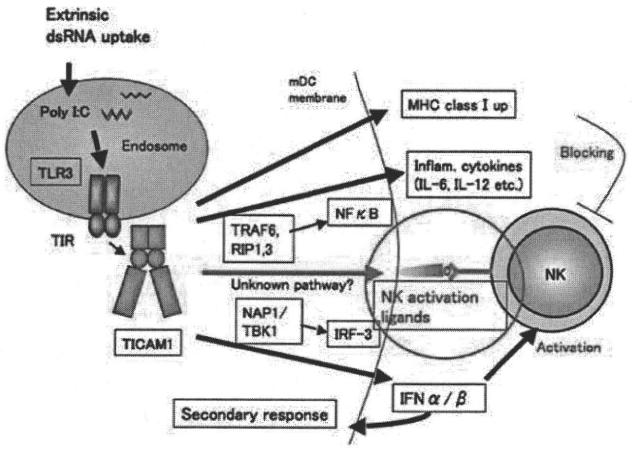

Fig. 4 Possible NK-inducing pathways against cancer. NK activation is an example of mDC output. For full activation, NK cells have to be supported by dendritic cells (myeloid DCs in this figure) that recognize pathogen-associated molecular patterns (PAMPs). In general, NK cells and dendritic cells are 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

effectively 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-γ 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 identification of several molecules as TICAM-1-dependent NK activation enhancers. NK activation followed by mDC maturation has a strong antitumor effect 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 effector 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 inflammation and are linked to smoldering inflammation around the tumor lesion to promote tumor incidence and growth [68]. The functions of CD4+ Th cells should be defined by discounting these Treg/Th17 effector functions.

The CD4+ Th cells consist of the Th1 and Th2 T cell subsets, based on their distinct cytokine secretion profiles. CD4+ Th1 cells produce cytokines IL-2 and IFN-γ. 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 differentiation 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 confirmed by another group [73]. Several attempts have been made to establish CD4+ T cell clones from tumor-infiltrating T cells. The results indicated that most CD4+ T cell clones are Th1 effectors that secrete IFN-γ 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-β and IL-6 produced by tumor cells, Treg cells and APCs activate the TGF-β and Stat3 signaling pathways, leading to the expression of RORγt, a critical transcription factor for Th17 cells [77]. Th17 cells were first identified 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 defined than that of Th1 cells. Nonetheless, both IL-17 and IL-23 have been identified in cancer tissues [78], suggesting that Th17 cells together with proinflammatory 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 identified 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, Tγ-1 and/or Th3 cells, are involved in T cells regulation [81]. Tγ-1 cells secrete IFN-γ and IL-10, while Th3 cells secrete high levels of TGF-β, IL-4 and IL-10. Foxp3 has been shown to be a specific 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 effects 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 different human cancers, including lung, breast and ovarian tumors [85, 86]. Ag-specific CD4+ Treg cells are situated at tumor sites, and these cells suppress the proliferation of naïve CD4+ Th cells upon activation by tumor-specific 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-infiltrating γδ T cells [89]. Naturally occurring Treg cells require the TICAM-1 pathway in Treg and mDCs for migration to inflamed 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 inflammatory Th1-type immunity is modulated by induced Treg cells, which also require the TICAM-1 pathway in mDCs [92]. Treg cells infiltrate the tumor mass and exert immunosuppressive effects 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 inflammation for danger signal

PAMPs usually trigger initial or early inflammation around tumors and immune cells in an extrinsic fashion. When tumor cells are damaged through extrinsic inflammation, the destructed cells release cytosolic and nuclear constituents. Inflammation 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 inflammation [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 inflammation 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 significant increase of the local effect 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 effector 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 effectors. Results from studies on the dsRNA recognition system, indicated that the properties of PAMP and repertoires of host receptors critically affect these processes. Another issue is how most effective effector cells are induced in a case-dependent manner for tumor remission in patients. This review provides guidelines for the development of specific effector cells by selecting dsRNA receptors. Current knowledge on the TICAM-1 pathway could be directly applied to cancer immunotherapy.

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Dendritic Cell/NK Cell Interaction in RNA Virus Infection

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Abstract: Natural killer (NK) cells constitute a major component of the innate immune system and have key roles in early immune responses to pathogens. Although NK cells can be directly activated by pathogens, other sensor cells which recognize pathogen-associated pattern molecules are required for the full activation of NK cells. The NK-activating capacity is observed in myeloid dendritic cells (mDCs), plasmacytoid DCs, macrophages and monocytes. The tropism of the pathogen and the route of invasion influence which sensor cells participate in the NK activation. Influenza virus, measles virus and respiratory syncytial virus can infect human mDCs and induce the ligands of the NK activating receptor NKG2D. Up-regulated NKG2D ligands on mDCs contribute to the mDC-mediated NK activation. By contrast, HCV does not replicate in mDCs and is not an immunostimulatory agent against mDCs. In this case, mDCs are stimulated after detecting dsRNA in the HCV-infected apoptotic hepatocytes *via* TLR3 and elicit NK activation though a direct cell/cell contact. The TLR3 signal seems to lead the up-regulation of key molecules on the surface membrane of mDCs to enhance NK activity by direct linkage.

Keywords: Dendritic cell, NK cell, RNA virus, hepatitis C virus, dsRNA, Toll like receptor 3.

INTRODUCTION

The NK cell is an important population of lymphocytes that have various effector functions against microbes and tumor invasion [1]. NK cells are able to kill tumor and infected cells *via* cytolytic granules and produce cytokines such as IFNg and TNFa to mediate the inflammatory response against 'non-self' objects. Unlike T cells and B cells, NK cells do not induce somatic recombination during their development. NK cells have many inhibitory and activating receptors, which are encoded in the germ-line [1]. The activity of NK cells is believed to be determined by the balance of activating and inhibitory signals. Since many inhibitory receptors recognize MHC class I molecules, NK cells exert cytotoxicity against MHC-class-I-deficient cells such as tumor cells and virus-infected cells. This is a so-called 'missing-self theory' [2].

In addition, NK cells are armed with many activating receptors. It has been reported that approximately twenty receptors contributed to NK activation [1]. Among them, NKG2D is the best characterized receptor. In humans, seven NKG2D ligands (UL 16 binding protein (ULBP) 1, 2, 3, 4 and 5 and MHC class I-related chain A and B (MICA/B)) have so far been reported [3]. Mice lack MIC genes but express the ULBP homologous proteins, namely RAE-1 (a, b, c, d, e), H60 and MULTI-1 [4]. NKG2D ligands exhibit highly restricted expression in healthy tissues, but they are widely expressed on tumor cells and infected cells [1, 3-5]. The expression of NKG2D ligands is induced by DNA damage, transformation, infection and heat shock [3]. However, in order to escape from the attack of NK cells, some viruses inhibit the expression of NKG2D ligands on the cell surface [6] and tumor cells secret soluble NKG2D ligands as decoys [7].

NK cells can be directly activated by viruses and ligands of Toll-like receptors (TLR) [6, 8-10]. Murine NK cells are known to recognize the cytomegalovirus-encoded m157 by its activating receptor Ly49H [11, 12]. Poly I:C is a synthetic dsRNA which is recognized by TLR3 and other cytosolic receptors. Poly I:C and unmethylated CpG DNA (TLR9 ligand) trigger secretion of IFNg from NK cells in the presence of IL-12 [8]. R848 (TLR7/8 ligand) also stimulates NK cells to produce IFNg [9, 10]. Although this direct NK activation works *in vitro*, several lines of evidence showed that the other sensor cells are required to recognize danger signals and that they strongly promote NK cytotoxicity and IFNg production [13-15]. The sensor cells in this context are myeloid dendritic cells (mDCs), plasmacytoid DCs (pDCs), macrophages and monocytes [15]. Here we summarize previous reports on DC-mediated NK activation and focus mainly on the role of mDCs to support NK activity in RNA virus infection.

NK CELL ACTIVATION THROUGH DENDRITIC CELLS

How DCs Sense Virus-Derived RNA?

Dendritic cells (DCs) are largely divided into two lineages, mDCs and pDCs [16]. mDCs are thought to be the dominant antigen presenting cells that activate naïve T cells. Although the main function of pDCs is to produce type I IFN, pDCs can present antigens to T cells. Both lineages of DCs develop different systems for sensing virus-derived RNA and initiating anti-RNA virus process, such as secreting type I interferon (IFN) and inflammatory cytokines (Fig. 1). pDCs express TLR7, which detects ssRNA in the endosome [17]. mDCs have retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated (MDA5) in the cytosol and TLR3 and TLR8 in the endosome [18-21].

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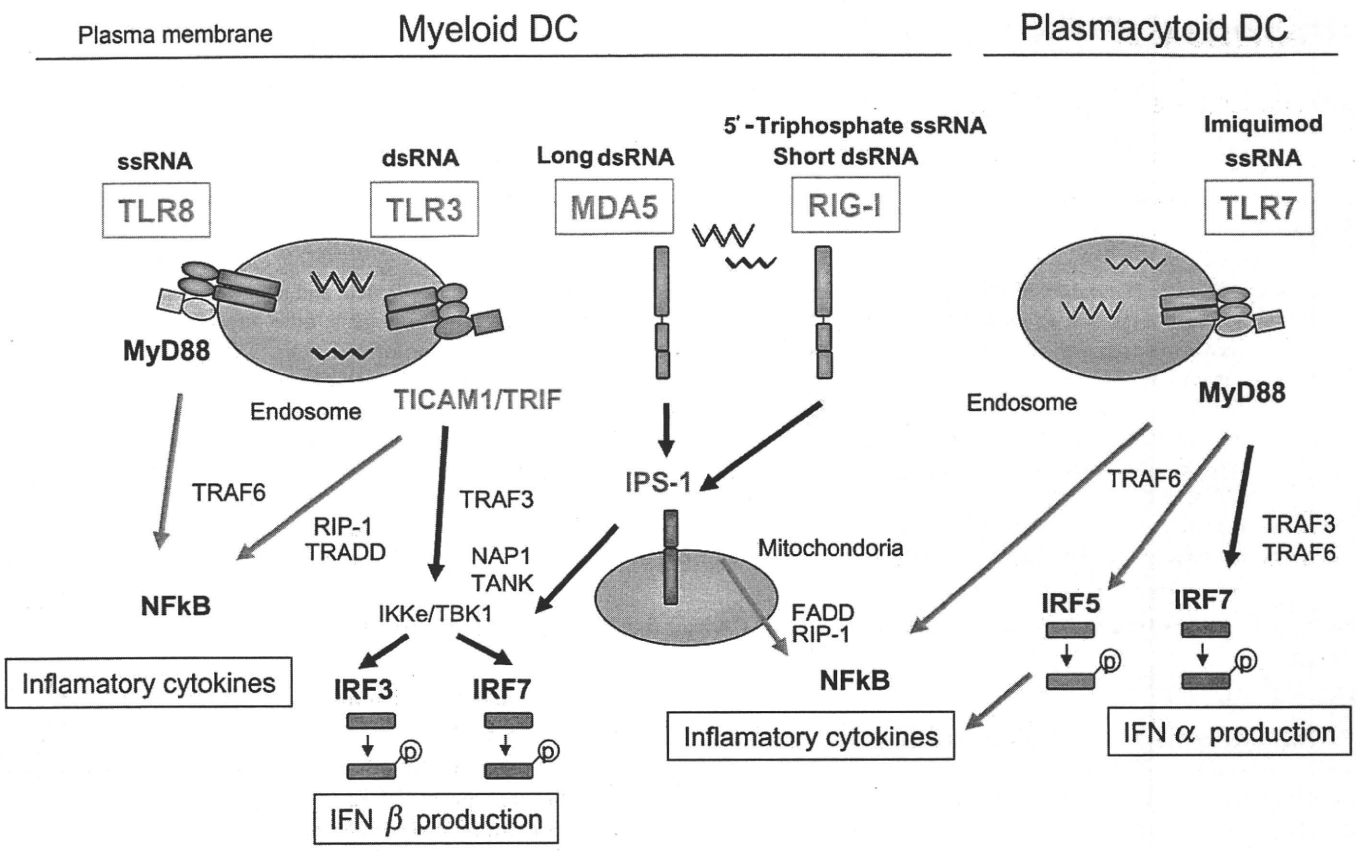

Fig. (1). How DCs sense virus-derived RNA. Myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) use different systems for defense against virus-derived RNA. pDCs express TLR7 in their endosomes and produce IFNa through IRF7 activation after recognition of virus-derived ssRNA. mDCs have MDA5 and RIG-I in the cytosol and TLR3 and TLR8 in the endosomes. MDA5 and RIG-I detect virus-derived RNA (MDA5: long dsRNA, RIG-I: short dsRNA and 5'-triphosphate ssRNA) in the site where non-self RNA is produced in virus-infected cells. Extrinsic dsRNA is recognized by TLR3 in the endosomes. The MDA5/RIG-I and TLR3 signal pathways lead to activation of IRF3 and IRF7, resulting in IFNb production. While TLR8 can participate in innate immunity by detecting extrinsic ssRNA, the role of TLR8 in mDC is not determined in actual RNA virus infection.

Whereas RIG-I, MDA5 and TLR3 sense dsRNA, they differentially recognize non-self RNAs. Since TLR3 resides in the endosome, dsRNA must enter endosomes for TLR3 signaling. There is no evidence that virus-derived dsRNA is moved from cytosol into the endosomes in virus-infected cells. Therefore, dsRNA has to be taken up extrinsically by endocytosis to encounter TLR3. Therefore, TLR3 has been thought to play a role in the defense against extrinsic dsRNA.

In contrast, intracellular cytosolic dsRNA is recognized by RIG-I and MDA5, which are widely distributed in tissues besides mDCs to circumvent attack from RNA viruses [19]. While MDA5 selectively recognizes long dsRNA (over 3k bp of poly I:C), short dsRNA (under 300bp of poly I:C) can activate RIG-I but not MDA5 [22]. RIG-I also detects the 5'ppp end of RNA generated by viral polymerases [23].

MyD88 is the adaptor protein for TLR7 and TLR8. Meanwhile, TLR3 recruits TICAM1/TRIF for its signaling [20,24]. In TLR7 signaling, MyD88 transduces signals to IRF5 and IRF7, which induce inflammatory cytokines and IFNa, respectively [25]. MyD88 also recruits the IL-1R-associated kinase (IRAK) family 1 and 4 and TNF-receptor-associated factor 6 (TRAF6), which promotes downstream activation of the IkB kinases (IKK), IKKa and IKKb by ubiquitination of IKKg. The IKKs directly phosphorylate members of the inhibitory IkB family, resulting in activation of NF-kB [20,24,25]. NF-kB is a ubiquitous transcription factor which is important for inflammatory cytokine expression. Although TLR8 is expressed in mDCs and pDCs in mice, murine TLR8 is non-functional [20,25]. TICAM1 is associated with receptor interacting protein kinase 1 (RIP-1) and TRAF6, which induce NF-kB activation. TICAM1 is associated with TBK1 (TRAF-family-member-associated NF-κB-activator-binding kinase 1) through NAK-associated protein 1 (NAP1) and possibly TRAF3 [26,27]. TBK1 forms a complex with IKKe and phosphorylates IRF3 and IRF7 to induce type I IFN production. RIG-I and MDA5 transduce signals to the same adaptor protein, interferon promoter stimulator 1 (IPS-1, also known as MAVS, VISA and Cardif) on the mitochondrial outer membrane [28-31]. The IPS-1 signal and the TLR3/TICAM1 signal converge upon NAP1 to activate TBK1 and IKKe, which contributes to type I IFN induction. IPS-1 also activates NF-kB *via* FADD- and RIP1-dependent pathways [32].

RNA Viruses that can Infect mDCs and pDCs

RNA viruses usually target mDCs and/or pDCs (Table 1). The capacity for type I IFN production was examined in mDCs and pDCs after incubation with RNA viruses mainly

in vitro. These *in vitro* data suggest that pDCs preferentially produce a type I IFN in response to many RNA virus infections compared to mDCs (Table 1). However, the route of infection seems to be crucial for determining which cell types play a main role in the production of type I IFN. Mouse mDCs produce a high level of IFNa comparable with pDCs in *in vitro* infection with Newcastle disease virus (NDV) [33]. An *in vivo* study showed that mouse splenic pDCs, as well as mDCs, monocytes and macrophages in the spleen and other organs, produce IFNa after intraperitoneal infection of NDV. However, in case of intratracheal NDV infection, IFNa production was detected in alveolar macrophages and mDCs, but not pDCs. On the other hand, measles virus (MV) infects large numbers of mDCs ($CD11c^+$/MHC class II^+) in peripheral tissues after intratracheal infection in Macaque monkeys [40]. Similar results were obtained with human CD46/CD150-transgenic mice, which express human MV receptors CD46 and CD150 [39]. Since the type I IFN induction by DCs is usually concordant with the infectivity of DCs, mDCs may play a dominant role in type I IFN production during MV infection.

pDCs can detect inactivated or live RNA viruses by TLR7 [35]. pDC responses occur against heat-inactivated or formaldehyde-fixed influenza virus *via* TLR7 and can induce IFNa comparable to their live counterparts. However, pDCs

Table 1. RNA Viruses that Target Dendritic Cells

Virus	Genome	RNA Sensor	Dendritic Cells	Main Type I IFN Producer	References
Paramyxoviridae					
Newcastle Disease Virus	(-)ssRNA	TLR7	Mouse pDC	pDC, mDC, macrophage	[33, 34]
		RIG-I	Mouse mDC		
Sendai Virus DI particle	(-)ssRNA	TLR7	Mouse pDC	ND	[35-37]
		MDA5	Mouse and human mDC		
Measles Virus	(-)ssRNA	ND	Human pDC	mDC?	[38-40]
		RIG-I/MDA5	Human mDC		
Respiratory syncytial virus	(-)ssRNA	ND	Human pDC	pDC	[38, 41, 42]
		RIG-I	Human mDC		
Rhabdoviridae					
Vesicular stomatitis virus	(-)ssRNA	TLR7	Mouse pDC	pDC	[35, 43, 44]
		RIG-I	Mouse mDC		
Orthomyxoviridae					
Influenza virus	(-)ssRNA	TLR7	Mouse pDC	pDC	[35, 45, 46]
		RIG-I	Mouse mDC		
Picornaviridae					
Encephalomyocarditis virus	(+)ssRNA	TLR7	Mouse pDC	pDC	[47, 43]
		MDA5	Mouse mDC		
Flaviviridae					
Dengue virus	(+)ssRNA	TLR7	Human pDC	ND	[48, 49]
		ND	Human mDC		
Retroviridae					
Human immunodeficiency virus	(+)ssRNA	TLR7	Human pDC	pDC	[50, 51]
		ND	Human mDC		
Coronaviridae					
Coronavirus (MHV, SARS)	(+)ssRNA	TLR7	Mouse and human pDC	pDC	[34]
Caliciviridae					
Murine norovirus-1	(+)ssRNA	MDA5	Mouse mDC	ND	[52]
Rotaviridae					
Rotavirus	dsRNA	ND	Human pDC	pDC	[53]
		ND	Human mDC		

cannot produce IFNa against some viruses, such as respiratory syncytial virus (RSV) and vesicular stomatitis virus (VSV), if they are inactivated by UV-irradiation. In this case, autophagy is required for the production of IFNa by pDCs [35]. Cytosolic viral replication intermediates are transported to the lysosome *via* autophagy and encounter TLR7.

In RNA virus infection, the function of TLR8 in mDCs remains to be elucidated. There are no reports of the role of TLR8 following exposure to RNA viruses, which can infect mDCs and have potent TLR8-stimulating RNA sequences in their genome. Although some synthetic nucleoside analogs are selectively recognized by TLR8 [54], it is unknown what patterns of virus RNA are sensed by TLR8. Besides DCs, coxsackie B virus and human parechovirus 1 trigger inflammatory responses *via* TLR8 in human cardiac cells and airway epithelial cells, respectively [55, 56]. However, it is uncertain whether these two viruses can infect mDCs.

The Molecules that Regulate DC/NK Interaction

DCs reciprocally activate NK cells in DC/NK co-culture conditions (Fig. 2). Although the role of mDCs has been well described in this DC-mediated NK activation, there have been limited reports on how pDCs activate NK cells. Therefore, it remains to be undetermined which dendritic cells are mainly responsible for NK activation in RNA virus infection. Gerosa *et al.* showed that pDCs-stimulated with inactivated influenza A virus (FluA) induce NK cytolytic activity and CD69 expression and that the effects are dependent on type I IFN [57]. In HSV-1 (DNA virus) infection, IL-18 produced by pDCs is a crucial factor for pDC-mediated NK IFNg production [58]. Besides these cytokines, pDCs express glucocorticoid induced tumor necrosis factor receptor ligand (GITRL) in response to viruses (HSV and FluA) [59]. GITR is detected on activated NK cells and GITRL/GITR interaction leads to NK cell cytotoxicity and IFNg production.

mDCs provide soluble signals (cytokines) and direct signals (*via* cell/cell contact) to NK cells (Fig. 2). Type I IFN, IL-12, IL-18, IL-15 and IL-2 are potent activators of NK cell effecter function [15]. By contrast, NK cells secrete IFNg and TNFa, which mature DCs [15]. Among these cytokines, type I IFN is a critical mediator of NK activation in viral infections and dsRNA stimulation [13,14]. IL-15 is

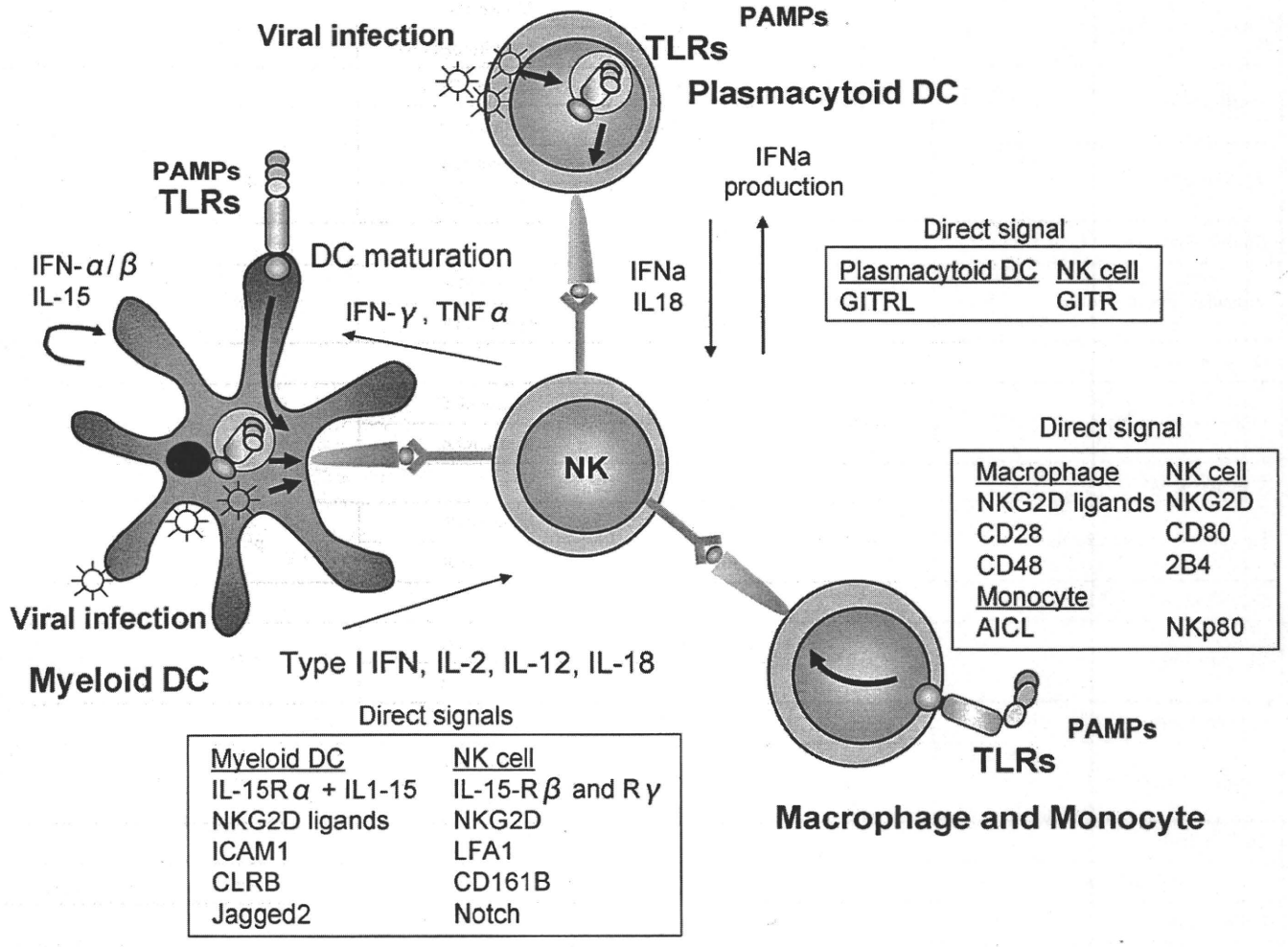

Fig. (2). Molecules that regulate the reciprocal interaction between sensor cells and NK cells. For full activation, NK cells have to be supported by sensor cells (myeloid DCs, plasmacytoid DCs, macrophages and monocytes, in this figure) that recognize pathogen-associated molecular patterns (PAMPs). In general, NK cells and sensor cells are reciprocally activated by soluble signals and cell/cell contact. Since the tropism of the pathogen is different from each other, the main NK activating players are determined by which sensor cells are attacked by the pathogens and induce innate signals for NK activation.

also a pivotal factor for the survival and proliferation of NK cells during co-culture with DCs [14]. Secreted IL-15 is immediately bound by IL-15 receptor-a expressed on DCs, and ligation of IL-15 receptor-b and g on NK cells causes increased cytotoxicity and IFNg production. IL-15 and IL-2 stimulate surface expression of lymphocyte function-associated antigen 1 (LFA1), which triggers degranulation and extracellular release of perforin and granzymes *via* activation of calcium-calmodulin kinase II in NK cells [60]. After MCMV (DNA virus) infection, NK cell cytotoxicity requires the interaction of NKG2D on NK cells with NKG2D ligands on mDCs [61]. The interaction of CLRB, C-type lectin-related molecule B, on mDCs and CD161B on NK cells enhances NK cytotoxicity after DC/NK co-culture [62]. CpG stimulates increased expression of Jagged2 on mDCs, which directly enhances NK cytotoxicity, IFNg production and cell proliferation *via* Notch on NK cells [63].

There is no evidence that macrophages and/or monocytes play a role in NK activation by RNA virus infection. LPS stimulation promotes CD48 expression on macrophages, and ligation of CD48 with 2B4 on NK cells leads to NK-cell proliferation and cytokine secretion [64]. Myeloid-specific activation induced C-type lectin (AICL) is up-regulated on monocytes by TLR ligands (TLR2, 3, 4, 8) [65]. The induced AICL promotes NK cell cytotoxicity through interaction with NKp80 on NK cells.

MDC/NK INTERACTION IN RNA VIRUS INFECTION

RNA Virus that can Infect mDCs

RNA virus infection usually enhances mDC maturation, which is characterized by up-regulation of CD80, CD83, CD86 and MHC class I and II molecules. Since MHC class I molecules are recognized by many NK inhibitory receptor [1], there must be some NK-activating ligands on mDCs, which overwhelm the inhibitory signal. Interaction of NKG2D and its ligands have been well described to cause strong NK activation. Therefore, expression of NKG2D ligands (ULBP1-5, MICA/B) was assessed on mDCs infected with RNA virus, such as FluA, MV, RSV and hepatitis C virus (HCV: the JFH1 strain) [66]. Transcription of ULBP2 and ULBP5 is induced in mDCs infected with all the RNA viruses except for HCV. This enhanced expression of ULBP2 and 5 is abrogated by UV-inactivation of these viruses [66], suggesting that RNA replication is required for up-regulation of ULBP2 and 5. RSV is a strong inducer of ULBP1 transcription. ULBP1 is known to be induced by a TLR4 ligand, LPS. In this case, RSV fusion protein might cause the elevated ULBP1 expression because RSV fusion protein stimulates mDC *via* TLR4.

These up-regulated NKG2D ligands on mDCs contribute to NK IFNg production. However, blockade of NKG2D ligands by anti-NKG2D antibodies leads to only a 20% decrease in NK IFNg production [66]. NKG2D ligands on mDCs have a limited role in mDC/NK cross-talk in RNA virus infections such as MV, FluA and RSV. In influenza virus infection, the mechanism of NK activation by mDCs has been further clarified. Draghi *et al.* showed that IFNa was mainly responsible for enhanced NK cytolysis and CD69 up-regulation, while IL-12 was necessary for enhancing IFNg production [67]. Enhanced CD69 expression and IFNg production, but not increased cytolysis, occurred *via* NK activating receptor NKp46 and NKG2D. NKp46 recognized influenza HA proteins expressed on the surface of infected-mDCs.

RNA Virus that Cannot Infect mDCs

As mentioned above, HCV (JFH-1 strain) does not induce expression of NKG2D ligands, in contrast to FluA, MV and RSV. This HCV strain was established by Wakita *et al.* and firstly reported as an infectious-particle which can propagate in an *in vitro* system [68]. Before this innovation, it was highly controversial whether HCV can replicate in mDCs, and inhibit the ability of mDCs to produce cytokines and stimulate allogenic T cells [69-72]. After incubation of mDCs wih the JFH1 strain, HCV core antigens and RNA replication are not observed in mDCs [73,74]. Therefore, mDCs are not permissive for HCV replication. In addition, NK cytotoxicity and IFNg production are not increased after coculture with mDCs inoculated with HCV. By contrast, FluA, MV and RSV are known to efficiently replicate in mDCs and stimulate mDCs to enhance NK activity. Not only mDCs but also pDCs are not infected with the JFH1 strain and cannot produce IFNa after exposure to this strain [73, 74]. Therefore, HCV is not an immunostimulatory agent for both mDCs and pDCs. All of these data indicate that there must be pathways other than direct virus interaction that promote the ability of DCs to activate NK cells.

The key molecule for the NK activation is dsRNA in HCV-infected apoptotic hepatocytes (Fig. **3**). Since HCV is a positive single stranded RNA virus, dsRNA is produced in HCV-infected hepatocytes. The more efficiently HCV replicates in hepatocytes, the more apoptosis occurs in the infected cells [73]. A number of apoptotic vesicles are produced from HCV-infected apoptotic cells and these vesicles serve as containers for dsRNA. After the uptake of these containers *via* lipid raft-dependent phagocytosis, TLR3 recognizes the dsRNA in the phagosome of the mDCs. Next, the mDCs mature and produce type I IFN and inflammatory cytokines. The dsRNA-containing apoptotic vesicles seem to be very efficient at carrying dsRNA to the endosomal compartment where TLR3 resides, because mDCs cannot respond to HCV-derived dsRNA if the dsRNA-containing vesicles are ruptured by a freeze-thaw cycle. The maturation of mDCs results in increased NK cytotoxicity but not IFNg production. Furthermore, the HCV-infected apoptotic vesicles modulate mDC function to promote Th1-dominant immunity in the Th1/T helper 2 balance. This enhancement of NK cytotoxicity is completely abolished by transwell insertion between the stimulated mDCs and NK cells. Therefore, cell/cell contact is indispensable for this mode of NK activation. It remains unsolved what molecules on mDCs are involved in this increased NK cytotoxicity.

dsRNA Induces NK-Activating Ligands on mDCs

The dsRNA-derived signaling pathway that leads to NK activation by mDCs has precisely examined using TICAM1/TRIF -/- mice [13]. *In vivo* administration of poly I:C causes increased NK cytotoxicity in wild-type mice, but not in TICAM1 -/- mice. While direct stimulation of poly I:C with NK cells is not associated with NK cytotoxicity, mDCs – but not macrophages or $CD8^+$ splenic DCs – enhance NK cytotoxicity after co-culture. This increased cytotoxicity

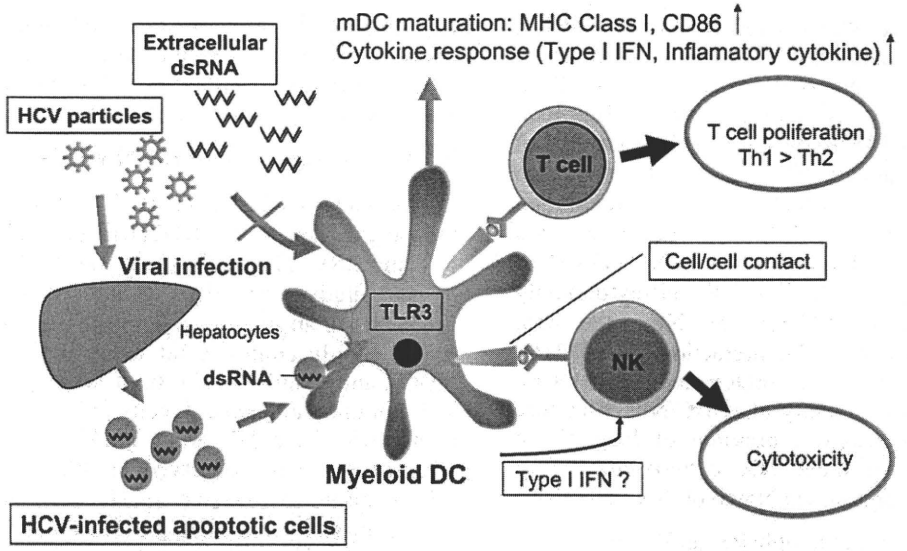

Fig. (3). HCV-infected apoptotic cells, but not HCV particles, regulate mDC function to activate NK cells and T cells. The HCV JFH1 strain does not directly stimulate mDCs or pDCs. In this case, pDCs and mDCs do not directly respond to the RNA virus. Our recent finding is that dsRNA in HCV-infected apoptotic vesicles is an immune-stimulator for mDCs that triggers the innate system for the activating T cell (Th1 dominant) and NK cells. Cell/cell contact between mDCs and NK cells is indispensable to increased NK cell cytotoxicity.

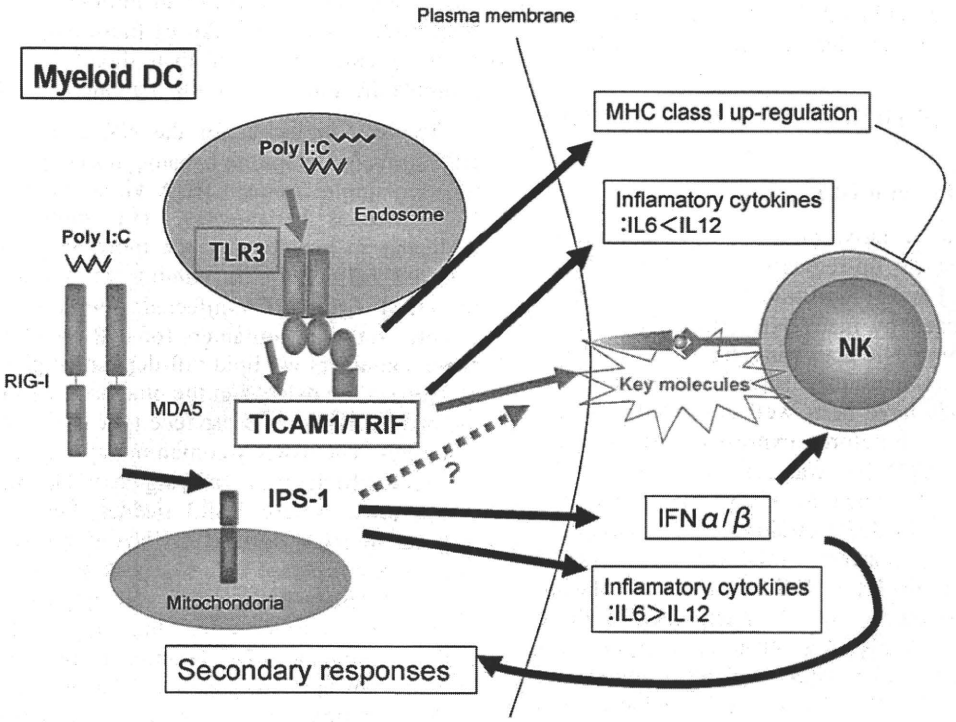

Fig. (4). The TLR3/TICAM1 signal leads to expression of unknown molecules on mDC, which activate NK cell *via* cell/cell contact. Among the three poly I:C receptors (TLR3, RIG-I and MDA5), the TLR3/TICAM1 signal plays an important role in regulating mDC capacity to activate NK cells. This mDC-mediated NK activation is dependent on the direct interaction of mDC and NK cells. Besides IL-15, there must be other key molecules that are induced on mDC by the TLR3/TICAM1 signal to activate NK cells because IL-15 is normally induced by poly I:C in TICAM-/- mice. NK activity is also regulated by type I IFN mediated by MDA5/IPS-1 pathway. Although IL-12 is produced mainly by the TICAM1 signal, IL-12 is not a functional entity that can enhance NK cytotoxicity by poly I:C-stimulated mDCs.

through poly I:C-stimulated DCs requires cell/cell contact. Although type I IFN is a key molecule regulating NK activation, the IFNa response in TICAM1 -/- mice is comparable to that in wild type mice. This is because another poly I:C receptor, MDA5, recognizes poly I:C and leads to IFNa production [19].

Poly I:C-dependent IL-12 production is impaired in TICAM1 -/- mice [13,19]. However, neutralization of IL-12 with anti IL-12 antibodies does not affect dsRNA-induced NK activation [13]. All of these data suggest that the TLR3/TICAM1 signal leads to up-regulation of a certain NK-activating ligand on mDCs. Lucas *et al.* showed that type I IFN secretion and trans-presentation of IL-15 by

mDCs are crucial to DC-mediated NK activation in response to poly I:C [14]. However, induction of IL-15 by mDCs is normal after stimulation with poly I:C in TICAM1-/- mice (unpublished data). We are now searching for the unknown molecules that regulate NK activation on mDCs (Fig. 4), other than IL-15. This unidentified factor may contribute to elucidation of the way NK cells are activated by mDCs during HCV infection.

CONCLUSION

NK cells are principal components of innate immunity and play a key role in early immune responses to viruses. The pathway mediating NK activation has been precisely examined *in vivo* by MCMV (DNA virus) infection. However, the receptors that sense virus-derived DNA are still unknown, except for TLR9 and DAI [75]. Many systems (such as TLR3, RIG-I, MDA5, TLR7 and TLR8) have been shown to trigger innate immunity against virus-derived RNA, but the mechanism of NK cell activation is less studied in RNA virus infection than in DNA virus infection. pDCs, mDCs, macrophages, monocytes and other cells invaded by RNA viruses presumably constitute a complicated network that activates NK cells. The tropism of RNA viruses appears to be the determinant of the mechanisms by which NK cells participate in innate immunity.

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