determined by ELISA.

Fig. 5. Rae-1 and MHC levels in the implant tumor of mice.

Mice were treated with anti-asialoGM-1 Ab, Spirulina, B16 tumor Ag and/or BCG-CWS as in Fig. 3, and inoculated with B16 cells, the prescriptions being indicated in the left column of the figure. five weeks later, the cells were harvested and dispersed in PBS/EDTA. The levels of Rae-1, MHC class I, MHC class II and Qa-1b were assessed by FACS using their specific Abs (16). Specific mean fluorescence intensities (MFI) of Rae-1 are indicated in the fluorograms as described in a previous report (16).

Fig. 6. Spirulina NK cells damage B16D8 tumor cells through MyD88 and NKG2D receptor.

A) NK cells are licensed to kill B16D8 cells through NKG2D after incubation with Spirulina-treated BMDCs. BMDCs from wild-type mice were stimulated with Spirulina extract for 4 h and mixed with NK cells for 24 h (DC:NK = 1:3). The mixture was incubated with anti-NKG2D Ab or control IgG and ^{51}Cr-labelled target B16 cells for 4 h at the E/T ratio indicated. B) MyD88 is crucial for Spirulina–mediated tumor growth retardation. Wild-type and MyD88 -/- mice were grouped as shown in the inset. Spirulina extract (600 μ /600 μl) (●, ■) or control saline (○, □) was orally administered to wild-type and MyD88 -/- mice (n = 5) every other day from day –14. B16D8 cells ($6x10^5$/head) were subcutaneously inoculated into the mice at day 0. Tumor volume was measured at indicated timed intervals and statistical analysis was performed as in Fig. 3.

Fig. 1

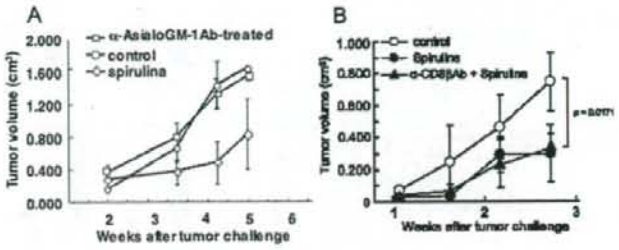

Fig. 1
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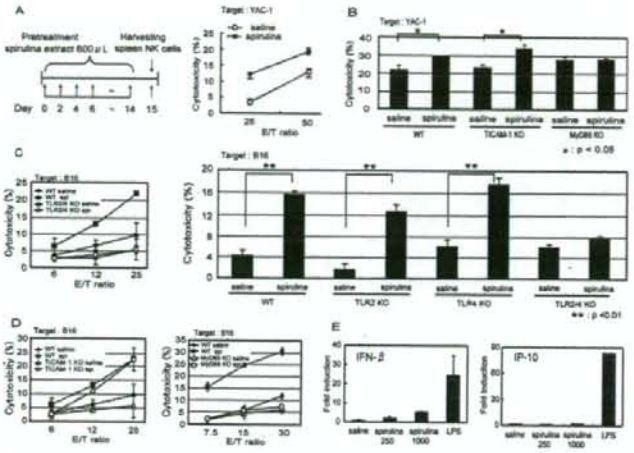

Fig. 2

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Fig. 3a

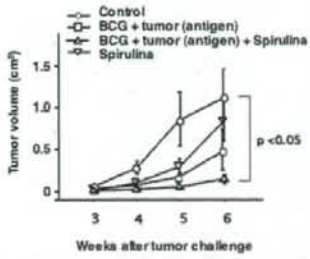

Fig. 3b

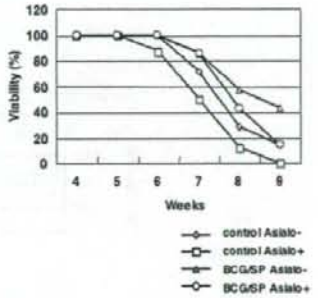

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Fig.4

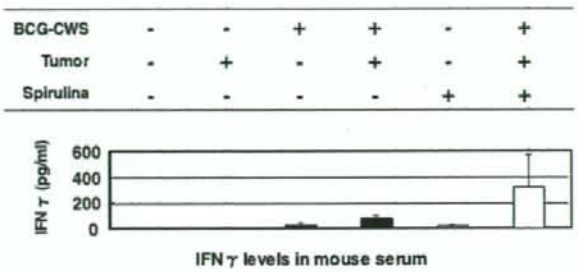

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Fig. 5

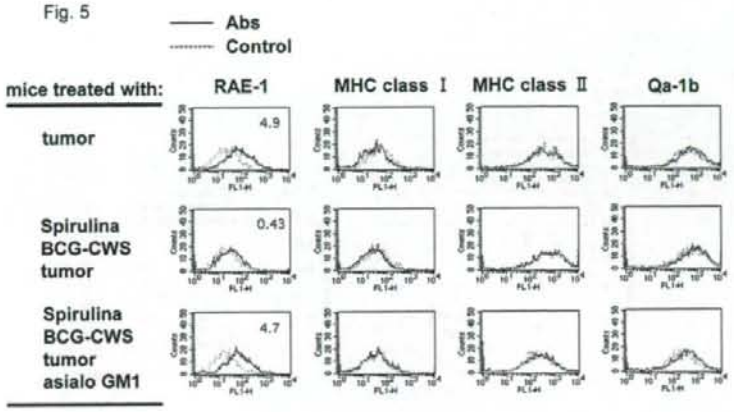

Fig. 5
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Fig. 6

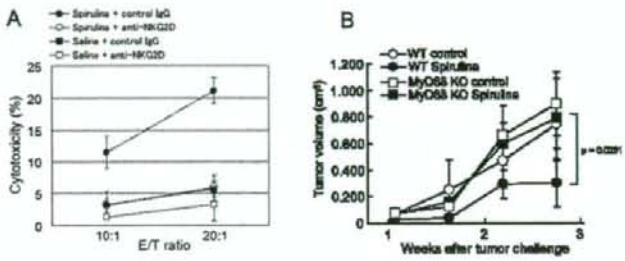

Fig. 6
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Dendritic cell/NK cell interaction in RNA virus infection

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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.

Key words: 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 tumours and in 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]. dsRNA (poly I:C, synthetic dsRNA) and unmethylated CpG DNA enhance NK activity in the presence of IL-12 by TLR3 and TLR9, respectively [8]. R848 (TLR7/8 ligand) also stimulates IFN-gamma production by NK cells [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 myeloid dendritic cells to support NK activity in RNA virus infection.

NK CELL ACTIVATION THOROUGH 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 [18-20]. Whereas RIG-I, MDA5 and TLR3 sense dsRNA, they differentially recognize non-self RNAs. Since TLR3 resides in the endosome, dsRNA must enter endosome for TLR3 signaling. However, in virus-infected cells, virus-derived dsRNA was not proved to move into the endosome. Therefore, dsRNA has to be taken up by endocytosis to encounter TLR3. 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]. This 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-_B-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 *in vitro*. 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]. Mouse splenic pDCs, as well as cDCs, monocytes and macrophages in the spleen and other organs, produce IFNa in response to intraperitoneally injected NDV. In contrast, NDV infection in the mouse lung leads to IFNa production in alveolar macrophages and cDCs, but not pDCs. On the other hand, measles virus (MV) infects large numbers of mDCs ($CD11c^+$/MHC class II^+) in peripheral tissues after intra-tracheally 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 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 RNA sequences in their genome detectable by TLR8. 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.

Table 1. RNA viruses that target dendritic cells

Virus	Genome	RNA sensor	Dendritic cells	Main type I IFN producer	Reference
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		

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 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 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. 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. Since interaction of NKG2D and its ligands is well described and strongly stimulates NK activation, 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. RSV is a strong inducer only of ULBP1 mRNA. Surface expression of ULBP1 and 2 is increased by RNA virus infection.

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 first reported as an infectious-particle-propagating *in vitro* system [68]. Before this innovation, the notion that HCV can replicate in mDCs, and inhibit the ability of mDCs to produce cytokines and stimulate allogenic T cells, was highly controversial [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 by mDCs inoculated with HCV. By contrast, FluA, MV and RSV are known to efficiently replicate in mDCs and stimulate mDCs to enhance NK activity. Furthermore, induction 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. 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 is outside the vesicles. 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. Thus, NK activation cannot be observed 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 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 produce IFNa [19].

PolyI: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 or factors 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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