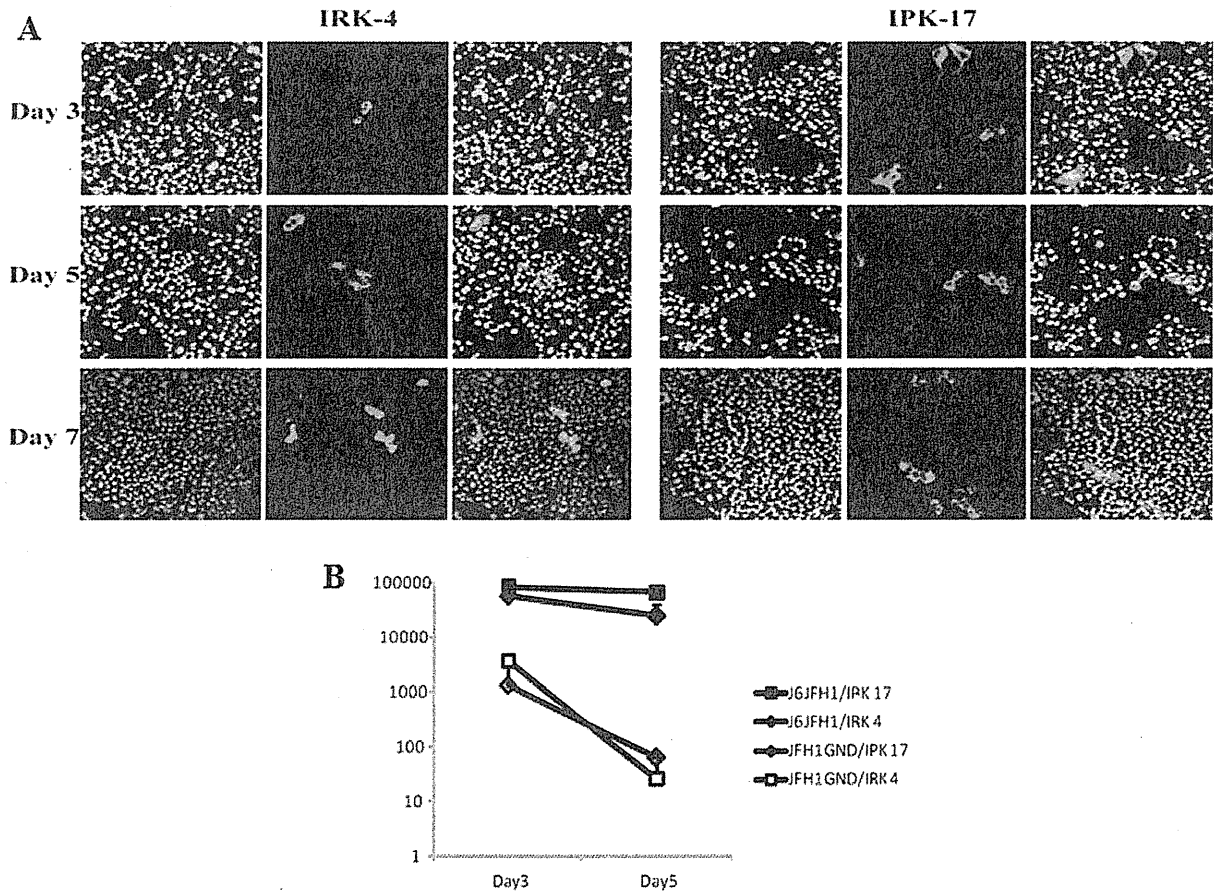

Figure 3. Proliferation of HCV in IRK4 and IPK17 cells over time as detected by immunofluorescence staining of NS5a protein using the CL1 rabbit polyclonal antibody (A) and by quantitative real-time RT-PCR analysis of HCV-RNA levels (B). JFH1GND was used as a negative control to exclude non replicating HCV-RNA. The data plotted represent the average +/− STD of 3 different experiments.
doi:10.1371/journal.pone.0021284.g003

Discussion

Gene silencing of either IPS-1 or IFNAR significantly improves HCV replication and persistence in mouse hepatocytes compared with wild-type or TICAM-1ko mice. This result demonstrated the importance of the IPS-1 pathway rather than the TICAM-1 pathway in the induction of type I IFN by HCV infection, and revealed that the IFNAR amplification pathway confers resistance to HCV in mouse hepatocytes independently of TICAM-1. In accordance with our data, HCV-NS3/4A protease is known to cleave the IPS-1 and/or RIG-I-complement molecules including DDX3 and Riplet in humans to overcome the host innate immune response, showing the importance of RIG-I/IPS-1 pathway suppression in the establishment of HCV infection [10,11,12].

To further study factors affecting the HCV life cycle in mouse hepatocytes, we established IPK and IRK immortalized mouse hepatocyte lines by transduction with SV40T antigen. The established hepatocytes cell lines showed expression of HNF4, a major hepatocyte transcription factor, required for hepatocyte differentiation and liver-specific gene expression [13]. The maintenance of hepatocellular functions was demonstrated by continuous expression of hepatocyte specific differentiation marker, albumin, and the lack of expression of the bile duct marker, cytokeratin. The close resemblance of these cell lines to primary mouse hepatocytes is crucial to ensure the physiological relevance of factors identified in these cell lines that affect the HCV life cycle.

It is worth noting that HCV replication in IPS-1ko was higher than that in IFNARko hepatocytes. Since IPS-1 is present upstream of IFNAR in the IFN-amplification pathway, this higher J6JFH1 replication efficiency in IPS-1ko hepatocytes suggested the presence of an additive factor affecting HCV replication other than the induction of IFNAR-mediated type I IFN. This enhanced replication efficiency was also not accompanied by the induction of other interferon types, but was correlated with the reduction of HCV-induced apoptosis in mouse hepatocytes. This data clearly demonstrates that IPS-1 is playing an important role in the regulation of HCV infection in mouse hepatocytes through two different pathways, the IFN-induction pathways and another new IFN-independent pathway, leading to apoptotic cell death and elimination of HCV-harboring hepatocytes. The cytopathic effect of HCV infection in human cells is still contradictory. Although, some reports showed the induction of apoptosis and cell death by HCV infection in human hepatocytes [14,15,16], others showed suppression of apoptosis by HCV proteins [17,18]. This difference may be due to the different cell lines used in the different studies. Almost all the studies reporting HCV-induced apoptosis used

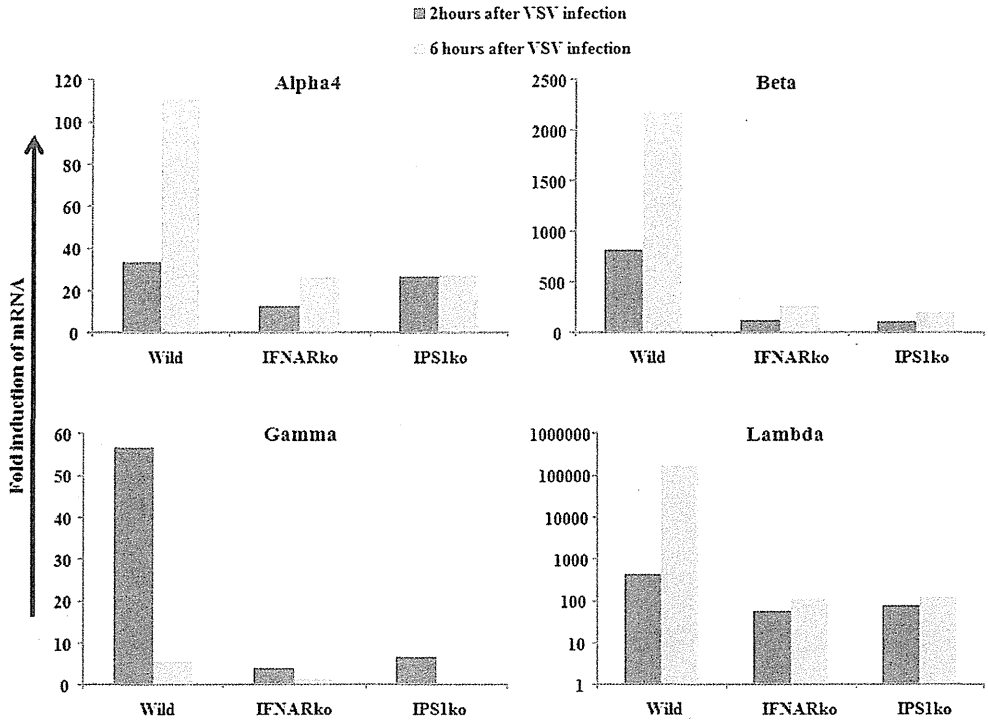

Figure 4. Wild type, IFNARko, and IPS-1ko mice hepatocytes were infected with mock or VSV virus, 2 and 6 hours later, total RNA was extracted from the cells, and interferon alpha, beta, gamma and lambda mRNA induction levels were measured by real-time RT-PCR. Similar results were obtained from 2 different experiments, each was performed in duplicates. The data plotted represent the mean duplicate readings in one of them.
doi:10.1371/journal.pone.0021284.g004

hepatocellular carcinoma cell lines. Since it has been established that the inability to undergo apoptosis is essential for the development of cancer [19,20,21], our use of immortalized, non-cancerous hepatocytes may make it possible to reproduce the physiological response of the cells to HCV infection more closely. The IPS-1 regulation of cell death following the introduction of HCV-RNA may also regulate the effector cell function. It is likely that hepatocyte debris generated secondary to intrinsic production of viral dsRNA in HCV-infected hepatocytes affect the antiviral effector response of the immune system through maturation of dendritic cells [22]. Hence, the effector cell activation may be enhanced by the induction of cell death through the IPS-1 pathway in hepatocytes which may facilitate producing dsRNA-containing debris.

In comparison to the JFH1GND construct with deficient replication that showed a rapid reduction in its RNA levels over time after transfection into mouse hepatocytes, J6JFH1 RNA was detected at four-log higher levels and was maintained at a relatively stable levels in IPS-1ko hepatocytes. Although the number of mouse cells expressing HCV proteins was found to increase over time, as detected by IF, the ratio between HCV-negative and -positive cells did not show any significant change for 7 days after transfection and increased after 10 days (data not shown). This indicates a negative selection of HCV-bearing cells over time which may be due to slower cellular replication, or loss of HCV replication. Another possibility may be that HCV infection is affected by the presence of an inhibitory factor possibly triggered by HCV replication or the lack of a human host factor required for HCV replication. Due to the initial replication of HCV in the transfected IPK and IRK mouse hepatocytes for the first 7 days and the establishment of infection, we favor the presence of a possible inhibitory factor that may be triggered by HCV replication. Another factor that also limits HCV spread in mouse hepatocytes is the failure of HCV to produce infectious particles in these cells (data no shown).

Using this newly established immortalized mouse hepatocyte line, we found that although J6JFH1, JFH1FL and the subgenomic JFH1 replicon all share a similar non-structural region derived from isolate JFH1 that is required for HCV replication, and although all of these constructs can replicate efficiently in HuH7.5.1 cells, strikingly, only J6JFH1 carrying the J6 structural region replicated in mouse hepatocytes. This indicates the importance of the J6 structural region and/or the chimeric construct between J6 and JFH1 for HCV replication in mouse hepatocytes. Structural regions are known to be important for HCV entry and/or particle formation [23], but this is the first time that their importance in replication in HCV-bearing cells has been demonstrated. This finding clearly shows the importance of non-hepatoma cell lines with less genetic abnormalities and mutations for the discovery of new aspects of the life cycle of HCV.

Although, the co-expression of human CD81 and Occludin genes was found to be important for HCVpp entry into murine NIH3T3 cells [3], the expression of hCD81 alone was sufficient for J6JFH1 entry into mouse hepatocytes. This may be explained by the different cell lines used in the different studies. In contrast to NIH3T3 cells, we used immortalized hepatocytes that showed close physiological resemblance to primary mouse hepatocytes and showed the expression of all the mouse counterparts of HCV entry

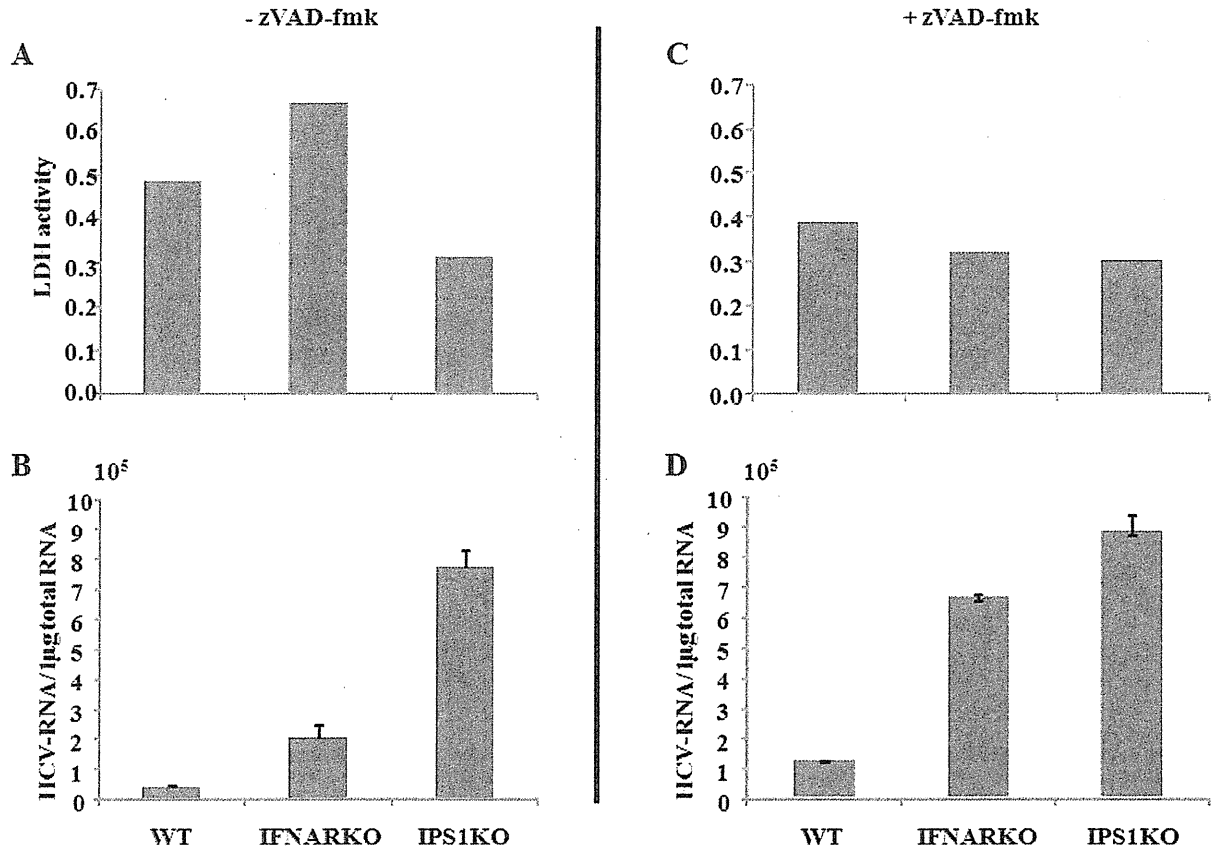

Figure 5. Measurement of J6JFH1 mediated cytopathic effect in wild type, IFNARko, and IPS-1ko mouse hepatocytes. Cultrure medium were left untreated (A;B) or treated with 20 μM of zVAD-fmK (C;D) 2 days before and after J6JFH1-RNA transfection. One day after transfection of J6JFH1-RNA, culture medium was discarded and cells were washed with PBS. A new medium was added and cells were cultured for another 24 hours. The LDH activity in the culture medium was measured in 2 different experiments in duplicates and showed similar results, the average levels of a duplicate from a single experiment was plotted (A, C). HCV-RNA titers in the cells were also measured using real-time RT-PCR (B, D), the data shown represent the mean +/− STD of 3 different experiments.
doi:10.1371/journal.pone.0021284.g005

receptors. A study from a different group showed that adaptive mutations in HCV envelope proteins allowing its interaction with murine CD81 is enough for efficient HCVpp entry without the expression of any human entry receptors in murine cells [24]. This report, together with ours, suggest that CD81 is the main human host restriction factor for HCV entry, and that overcoming this problem either by HCV adaptation to murine CD81, or the expression of human CD81 in murine hepatocytes is essential for HCV entry. Although our lentivirus transfection efficiency with CD81 was around 95% in IPK and IRK clones, only 1% of the cells were prone to infection with HCVcc. Also, HCVpp showed lower entry levels in those cells compared to HuH7.5.1 cells (Fig. S6). This suggests that hCD81 expression is the minimum and most crucial requirement for HCV entry into mouse hepatocytes. The discovery and expression of other co-receptors facilitating HCV entry in human cells is still required for efficient and robust HCV infection.

In summary, the suppression of IPS-1 is important for the establishment of HCV infection and replication in mouse hepatocytes through the suppression of both interferon induction and interferon independent J6JFH1-induced cytopathic effect. We have established hepatocytes lines from IPS-1 and IFNARko mice that support HCV replication and infection. These cell lines will be very useful in identifying other species restriction factors and viral determinants required for further establishment of a robust and efficient HCV life cycle in mouse hepatocytes. Using those cells, we showed for the first time the importance of HCV structural region for viral replication. IRF3ko mouse embryo fibroblasts (MEFs) were previously shown to support HCV replication more efficiently than wild MEFs [25]. Since the knockout of IPS-1 mainly suppresses signaling in response to virus RNA detection, and maintains an intact IFN response to other stimulants, it may result in minimum interference to adaptive immune responses as compared to IRF3 or IFNARko. Therefore, further development of hCD81-transgenic IPS-1ko mice may serve as a good model for the study of immunological responses against HCV infection. This mouse model can be used as a backbone for any further future models supporting robust HCV infectivity for the study of HCV pathogenesis, propagation and vaccine development.

Material and Methods

Cell culture

HuH7.5.1 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen, Tokyo, Japan) supplemented with 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg of

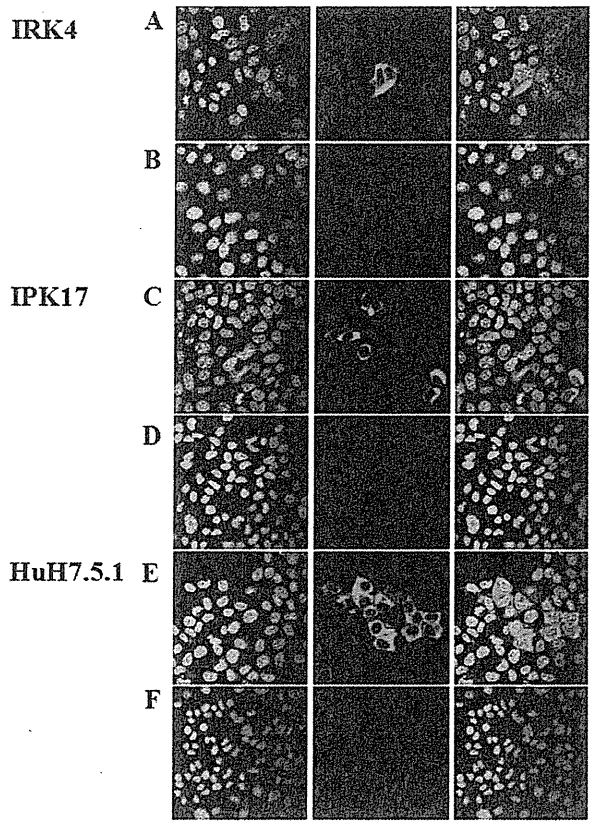

Figure 6. J6JFH1 infection into IRK-4 and IPK17 cells. HCV-NS5A protein detection in mouse IRK4 (A,B) and IPK17 (C,D) and human 7.5.1 cells (E,F). The cells were transduced with lentivirus expressing human CD81 gene at 10 MOI. 48 hours later the cells were infected with 100 times concentrated supernatant medium, collected during 1 week after transfection of HuH7.5.1 cells with J6JFH1-RNA (A, C, and E) or JFH1GND-RNA (B, D, and F).
doi:10.1371/journal.pone.0021284.g006

streptomycin/ml and 10% fetal bovine serum. Mouse primary hepatocytes were isolated from the liver using collagenase perfusion through the inferior vena cava (IVC), while clamping the animal's intrathoracic extension. Hepatocyte isolation and perfusion control were performed as previously described [26]. Primary and immortalized hepatocytes were cultured in a similar medium supplemented with: HEPES (Gibco/Invitrogen), 20 mmol/L; L-proline, 30 μg/mL; insulin (Sigma, St. Louis, MO, USA), 0.5 μg/mL; dexamethasone (Wako, Osaka, Japan), 1×10^{-7} mol/L; $NaHCO_3$, 44 mmol/L; nicotinamide (Wako), 10 mmol/L; EGF (Wako), 10 ng/mL; L-ascorbic acid 2-phosphate (Wako), 0.2 mmol/L; and MEM-non essential amino acids (Gibco/Invitrogen), 1%.

Gene-disrupted mice

All mice were backcrossed with C57BL/6 mice more than seven times before use. Toll-like receptor adaptor molecule 1 (TICAM-1) ko [27] and IPS-1ko mice [28] were generated in our laboratory (detailed information regarding the IPS-1 mice will be presented elsewhere). All mice were maintained under specific-pathogen-free conditions in the animal facility of the Hokkaido University Graduate School of Medicine (Japan).

RNA extraction, reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR

RNA was extracted from cultured cells using Trizol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol. Using 1 μg of total RNA as a template, we performed RT-PCR and real-time RT-PCR as previously described [29,30].

In vitro RNA transcription, transfection and preparation of J6JFH1 and Jfh1 viruses

In vitro RNA transcription, transfection into HuH7.5.1 or mouse hepatocytes, and preparation of J6JFH1 and JFH1 viruses, were all performed as previously reported [31]. RNA transfection into human and mouse hepatocytes was performed by electroporation using a Gene Pulser II (Bio-Rad, Berkeley, California) at 260 V and 950 Cap.

HCV infection

J6JFH1 and JFH1 concentrated medium were adjusted to contain a similar RNA copy number by real-time RT-PCR. 2×10^4 cells/well were cultured in 8-well glass chamber slides. After 24 hours, the medium was removed and replaced by concentrated medium containing JFH1 or J6JFH1 viruses. After three hours, the concentrated medium was removed, cells were washed with PBS and incubated in fresh medium for 48 hours, before the detection of infection.

Lentivirus construction, titration and infection

The gene encoding T antigen from simian virus was cloned from plasmid CSII-EF-SVT [32]. The genes encoding human CD81 and occludin were cloned from HuH-7.5.1 cells using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) according to the manufacturer's protocol. These genes were then inserted into the GFP reporter gene-containing lentiviral expression (pLBIG) vector using the *Eco*RI and *Xho*I restriction sites for SV40T and hCD81, and the *Xba*I and *Xho*I restriction sites for hOccludin. Lentivirus expression vectors were then constructed as previously described [27]. GFP expression was used for the titration of lentivirus vectors, and a multiplicity of infection (MOI) of 10 was used for the infection of mouse cells. Forty-eight hours after the transfection of hCD81 and/or hOccludin, cells were trypsinized and counted. Then, 2×10^4 cells/well were cultured in 8-well glass chamber slides for HCV infection and 5×10^4 cells/well were cultured in 12-well plates, along with 1 ml of medium containing HCVpp, for HCV entry experiments.

HCVpp construction and the detection of luciferase expression

HCVpp containing the E1 and E2 proteins from HCV isolate J6 and expressing the luciferase reporter gene were a kind gift from Dr. Thomas Pietschmann at the TWINCORE Center for Experimental and Clinical Infection Research, Germany. The production of HCVpp and the measurement of luciferase levels were performed as previously described [33].

Indirect immunofluorescence (IF)

IF expression of HCV proteins was detected in the infected cells using antibodies in the serum of chronic HCV patients or rabbit IgG anti-NS5A antibody (Cl-1) (both kind gifts from K. Shimotohno, Chiba Institute of Technology, Japan). Goat anti-human IgG Alexa 594 and goat anti-rabbit Alexa 594 (Invitrogen) were used as secondary antibodies, respectively. Fluorescence

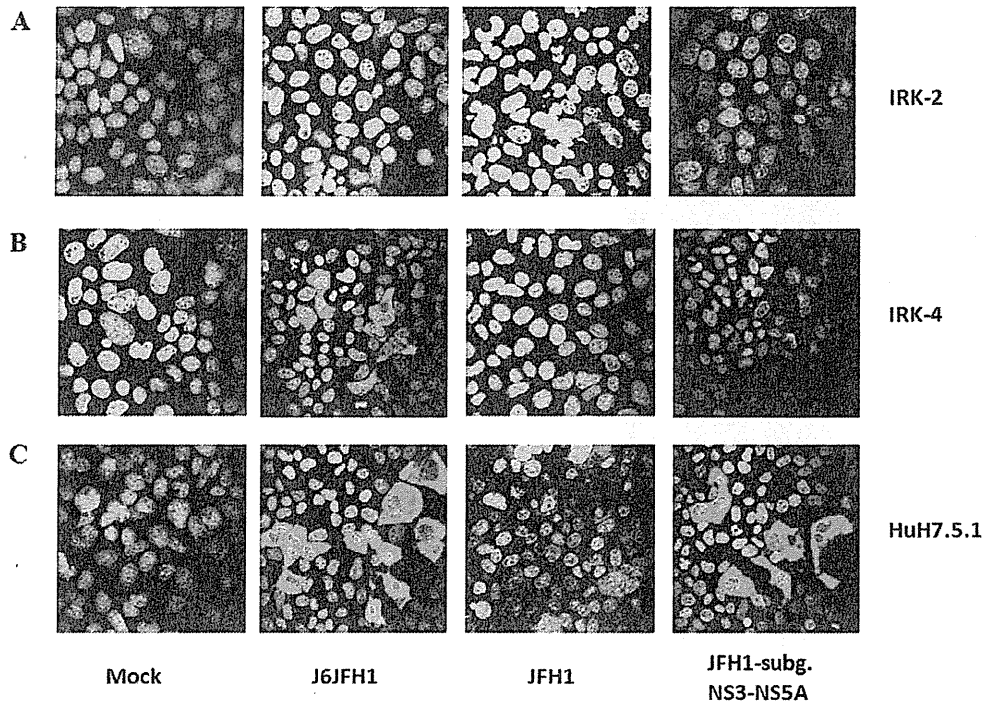

Figure 7. Detection of HCV-NS5A protein in IRK-2 (A), IRK-4 (B) and HuH-7.5 cells (C) by IF 5 days after transfection with J6JFH1, FL-JFH1 or subgenomic JFH1-RNA.
doi:10.1371/journal.pone.0021284.g007

detection was performed on a ZEISS LSM 510 Meta confocal microscope (Zeiss, Jena, Germany).

Detection of cell death

Culture medium was collected from HCV infected and control cells and used for measuring lactate dehydrogenase (LDH) levels using an LDH cytotoxicity detection kit (Takara Biomedicals, Tokyo, Japan). Light absorbance was then measured according to the manufacturer's protocol.

Ethic Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments in the Animal Safety Center, Hokkaido University, Japan. All mice were used according to the guidelines of the institutional animal care and use committee of Hokkaido University, who approved this study as ID number: 08-0243, " Analysis of immune modulation by toll-like receptors".

Supporting Information

Figure S1 RT detection of TLR3, TLR7, RIG-I, and IPS-1 expression in mouse hepatocytes. GAPDH expression was used as internal control, and RNA from CD11c+ spleenocytes (dendritic cells) was used as positive control.
(TIF)

Figure S2 Proliferation of HCV in IPS-1, TICAM-1(TRIF) and IFNAR-knockout mouse hepatocytes over time as detected by quantitative real-time RT-PCR analysis of HCV-RNA levels. JFH1GND transfection into IPS-1 knockout cells was used as a negative control to exclude non replicating HCV RNA. The data plotted represent the average +/− STD of 3 different experiments.
(TIF)

Figure S3 RT detection of CD81, Occludin, Claudin 1, SRB1, and LDL receptor expression in primary, IRK4 and IPK17 mouse hepatocytes. GAPDH expression was used as internal control.
(TIF)

Figure S4 Estimation of the transfection efficiency of lentivirus vector expressing green fluorescent protein (GFP) as a reporter, together with hCD81 or hOccludin. 48 hours after transfection with the lentivirus vector, cells were trypsinized and GFP positive cells were detected by BD FACSCalibur (BD Biosciences).
(TIF)

Figure S5 HCV infection of IRK2 cells transfected with lentivirus expressing hCD81 and/or hOccludin. IRK2 cells were transfected with lentivirus expressing empty vector (A), hCD81 (B), hOccludin (C) or hCD81 and hOccludin (D) at a MOI of 10. After 48 hours, the cells were infected with concentrated J6JFH1 transfected 7.5.1 culture medium. After a further three hours, cells were washed with PBS and incubated in fresh medium. After another 48 hours, HCV infection was examined through the detection of HCV-NS5a protein expression by immunofluorescence staining.
(TIF)

Figure S6 HCVpp entry into mouse cells. A similar number of IPK17 and HuH7.5.1 were cultured in triplicate. IPK17 cells were only transfected with lentivirus expressing hCD81, while HuH7.5.1 cells were transfected with empty vector at a MOI of

10. After 48 hours, the medium was replaced with a new medium containing mock VSVG-pp or HCVpp expressing luciferase. After another 48 hours, pseudoparticles entry was determined by measuring the luciferase activity. In order to compare the HCVpp entry between IPK17 and HuH7.5.1 cells, the luciferase expression from VSV-Gpp entry was used an internal control, while that from HCVpp was plotted relatively.
(TIF)

Acknowledgments

We want to thank Dr. Michinori Kohara (Tokyo Metropolitan Institute for Medical Science, Tokyo, Japan); Dr. Tadatsugu Taniguchi (University of Tokyo, Yokyo, Japan); Dr. Thomas Pietschmann (Division of Experimental Virology, TWINCORE, Hannover, Germany); and Dr. Makoto Hijikata (The Institute for Virus Research, Kyoto University, Japan) for their generous supply of research material. Dr. Hussein H. Aly was supported by a JSPS postdoctoral fellowship from the Japan Society for the Promotion of Science.

Author Contributions

Conceived and designed the experiments: HHA TS. Performed the experiments: HHA HO. Analyzed the data: HHA MM HO HS TS. Contributed reagents/materials/analysis tools: KS TW. Wrote the paper: HHA.

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Review

Journal of Innate Immunity

J Innate Immun 2011;3:264–273
DOI: 10.1159/000326891

Received: January 20, 2011
Accepted after revision: February 24, 2011
Published online: March 29, 2011

Natural Killer Cell Activation Secondary to Innate Pattern Sensing

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Key Words
Natural killer cell activation · Dendritic cell · Toll-like receptor 3 · TICAM-1 · MyD88

Abstract
Recent progress in understanding the outcomes of pattern-recognition by myeloid dendritic cells (mDC) allows us to delineate the pathways driving natural killer (NK) cell activation. Mouse mDC mature in response to microbial patterns and are converted to an NK cell-activating phenotype. The MyD88 pathway, the Toll/IL-1 receptor homology domain-containing adaptor molecule (TICAM)-1 (TRIF) pathway, and the interferon (IFN)-β promoter stimulator 1 (IPS-1) pathway in mDC participate in driving NK activation, as shown by analyses in knockout mice. Studies using synthetic compounds for Toll-like receptors/RIG-I-like receptors have demonstrated that mDC-NK cell contact induces NK cell activation without the participation of cytokines in mice. In vivo bone marrow transplantation analysis revealed that the IPS-1 pathway in nonmyeloid cells and the TICAM-1 pathway in mDC are crucial for dsRNA-mediated in vivo NK activation. These results infer the presence of cytokine-dependent and cytokine-independent modes of NK activation in conjunction with innate immune activation. Here, we focus on the IFN-inducing pathways and mDC-NK contact-induced NK activation and discuss the reported various NK activation modes.

Introduction

Natural killer (NK) cells have the ability to kill certain tumor cells and infected cells [1]. A number of activating and inhibitory receptors have been implicated in NK cell recognition and elimination of target cells [2]. In addition, NK cell effector functions are induced or potentiated through recognition of microbial products by innate pattern recognition receptors (PRRs) that are expressed in various cell types, including myeloid cells and NK cells. In this way, dendritic cells may induce cytokines, such as interferon (IFN)-γ, and potentiate cytotoxicity by NK cells [3]. In mice, myeloid cells stimulate NK cells through cell-cell contact and with soluble mediators [4, 5]. Many factors including cytokines and molecules supporting direct contact by immune-related cells are reported to participate in NK activation [2–5]. Myeloid dendritic cells (mDC) and macrophages (Mf) often serve as a source of such activating factors as IL-12p70, IL-18, IL-15, and type I IFN.

It is well known that type I IFN activates NK cells. However, immature mDC only subfunctionally produce type I IFN and, consequently, IFN-dependent NK activation factors are maintained at basal levels through the IFN-α receptor (IFNAR) pathway in mice [6]. Infection or inflammation stimulates additional factors that render NK activation by mDC feasible [4, 5]. Such factors that induce mDC maturation largely belong to a class of ex-

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ogenous or endogenous pattern molecules designated pathogen-associated molecular patterns (PAMP)/damage-associated molecular patterns (DAMP) [7, 8]. Research into signaling pathways in the innate immune system has indicated that PAMP and DAMP act on PRRs in mDC/Mf and drive NK activation [4, 5, 7, 8]. In addition, membrane molecules upregulated on the surface of mDC participate in NK activation in a process known as mDC-NK contact-mediated NK activation [4, 5]. In this case, mature mDC and NK cells must be recruited to local lymph nodes, and their interactions lead to the emergence of effector NK cells in the periphery. However, it is unknown whether activation of NK cells is totally dependent on IFN or just shares the PRR pathways with IFN induction to upregulate other NK-activating molecules. Furthermore, it remains undetermined what microenvironment mDC require for maturation along with NK activation and what effectors mDC stimulate via the PRR pathways to participate in enhancing NK activity.

Recent progress in the innate pattern-sensing system suggests that mDC pattern recognition is a major event in driving mDC to an NK-activating phenotype [9–11]. These results add new insight into the currently accepted theory that the balance between a number of activating receptors and inhibitory receptors and their activation states are critical for NK activation [2, 3, 12, 13]. Insight into the mechanism behind NK cell activation may be gained via analysis of the molecular mechanisms by which PAMP/DAMP activate the immune system and, in particular, mDC. NK cells have the capacity to induce memory-like responses in a way comparable to T lymphocytes [14], and some subsets are specialized to produce the Th17 cytokine IL-22 [15] although their features are not always comparable to NK cells. These unique features of NK cells may be associated with mDC factors that drive NK activation, including the combination of stimuli required for PRR and the cytokines that act in conjunction with inhibitory/activating ligands on NK receptors [16]. This review collates recent advances in the innate molecules and pathways related to mDC-mediated NK activation.

Direct or Secondary Activation of NK Cells by Microbial Patterns

DC/Mf as well as stromal cells express a variety of PRRs. In infection, these non-NK cell-derived mediators play a role in NK cell responses to pathogens [17]. Activation of these cells in response to PAMP can also lead to indirect NK activation which is mediated by affected accessory cells with altered membrane-associated molecules [16, 17]. Both soluble factors and membrane molecules join NK cell activation. Several reviews mention the mode of direct and accessory cell-derived (secondary) NK activation [2, 8, 16]. We just summarized this issue to facilitate the introduction of mDC/Mf-mediated NK cell activation.

Pathogen molecules often interact with NK receptors. Examples of direct pathogen interaction with NK cells have been demonstrated in mouse cytomegalovirus, whose m157 molecule interacts with Ly49H, an NK cell-activating/inhibitory receptor [18]. Influenza virus hemaglutinins bind the NKp46 of human NK cells [19]. Besides these NK receptor-interacting molecules, several kinds of bacteria/viruses are known to directly activate NK cells by PRR stimulation. Examples of microbial ligands for Toll-like receptors (TLRs) present in the NK cell membrane are as follows. Measles virus H protein interacts with TLR2 [20]. Mycobacteria muramyl dipeptides activate TLR2 residing on the NK cell membrane [21]. *Plasmodium falciparum* has an unidentified factor that interacts with TLR2/4 [22]. Some leishmania species have a lipophosphoglycan to bind to TLR2 [23]. Pam2 lipopeptides of a variety of bacteria serve as TLR2 ligands [24]. These factors also interact with mDC/Mf TLRs. Which TLRs in NK cells or accessory cells are more important for triggering NK cell activation in vivo should be an issue to be clarified.

Overview of the mDC Pattern Sensing System

mDC, which comprise many subsets [including bone marrow-derived DC (BMDC) and CD8α+ DC], possess subset-specific pattern-recognition systems. TLR, NOD-like receptors, and RIG-I-like receptors (RLR) are representative PRRs. The PRR repertoire in mDC has been described previously [17]. Two adaptors, i.e. MyD88 and TICAM-1 (TRIF), critically determine the signal pathways for TLRs, whereas interferon-β promoter stimulator 1 (IPS-1) (Cardif, MAVS, VISA) is the only adaptor that governs MDA5/RIG-I signaling (fig. 1). These adaptors are engaged in type I IFN induction and NK activation via partly overlapped but distinct pathways in a cell type-specific manner. Here, we summarize the signal pathways for TLR and RLR relevant to NK activation.

Signaling Pathways That Operate through the MyD88 Adaptor

MyD88 is the most common adaptor in the TLR and interleukin (IL)-1R signaling pathways [25, 26]. With the

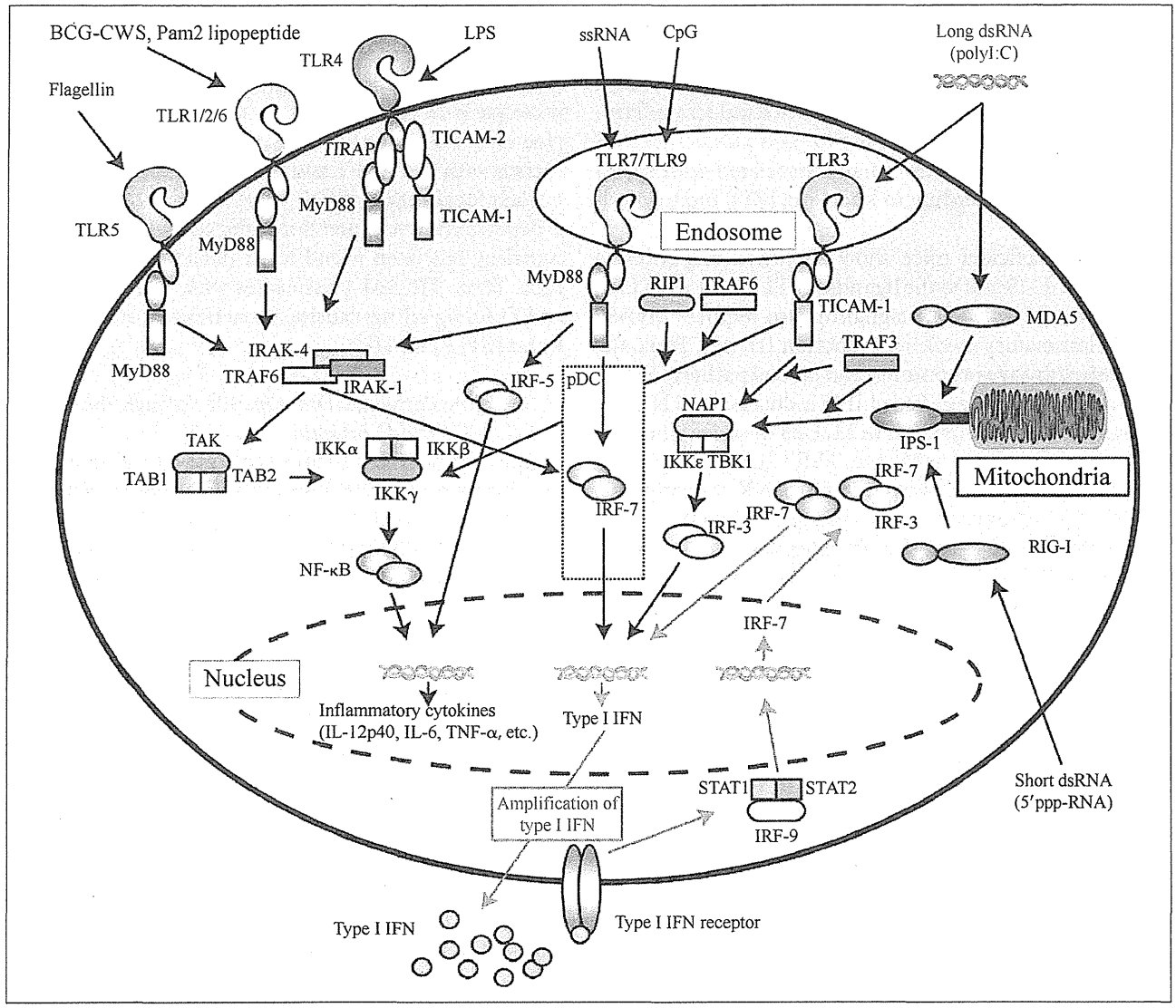

Fig. 1. TLR pattern-sensing system and cytoplasmic dsRNA recognition pathways. The TLR and RLR pattern-sensing systems involved in type I IFN induction and NK cell activation are depicted in a single cell model. pDC has a unique MyD88 pathway which is highlighted in the open square. All pathways except this MyD88 pathway function in BMDC of mouse and monocyte-derived DC of humans. Mouse spleen DC and human BDCA1/3-positive DC have weak potential for recognition of cytoplasmic dsRNA, reflecting a relatively immature feature of DC. Once TLR3 or TLR4 is stimulated, it transiently recruits TICAM-1 to form multimers. Subsequently, liberated TICAM-1 form a molecular complex named 'speckle' which serves as a nest for IRF-3/7 activation. In contrast, IPS-1 binds the outer membrane of mitochondria and forms an IRF-3/7-activating complex on the membrane. Once type I IFN is liberated, type I IFN production is amplified via the IFNAR pathway. NK cell activation occurs not only by soluble factors but also membrane proteins induced secondary to IRF-3 activation.

exception of TLR3, all TLRs in mice and humans can couple with MyD88. Only the MyD88 in plasmacytoid DC (pDC) is linked to IFN-induced signaling [27]. In pDC, TLR7/9 recruit MyD88 and the IRAK4/IRAK1 complex, allowing the phosphorylation of IRAK1 [28]. Activated IRAK1 forms a molecular complex with TRAF6/TRAF3/Osteopontin and IKKα. After forming a complex with MyD88, activated IKKα activates nearby IFN regulatory factor (IRF)-7 which, in turn, is dimerized and translocated to the nucleus [29]. The IRF-7 di-

mer then initiates transcription of the IFN-α gene. Once IFN-α is liberated, the IFNAR on the same cell or on other DC is activated to amplify the production of IFN-α [6, 28, 29]. MyD88 also recruits IRF-5 which induces inflammatory cytokines, IL-6, IL-12p40, and tumor necrosis factor (TNF)-α but is minimally associated with type I IFN induction according to knockout (KO) mouse studies [30] (fig. 1).

MyD88-deficient mice show abolished responses to IL-1β and IL-18 and to the ligands for TLR2, TLR5, TLR7, and TLR9 [17, 25]. TLR4 signaling also requires MyD88 for inflammatory cytokine induction [17, 25]. Thus, the MyD88 pathway is a common and pivotal pathway for IL-1R and TLR signaling. Type I IFN induction and NK cell activation are not impaired in MyD88–/– mice when the mice are stimulated with the TLR3 ligand polyI:C (a dsRNA analog) [9]. However, in vitro, NK activation via mDC TLR2 stimulation is abolished in MyD88–/– cells [24], as discussed later. The physiological importance of MyD88 in NK activation in vivo in a variety of TLR pathways remains to be proven.

Signaling Pathways That Operate through the TICAM-1 (TRIF) Adaptor

TLR3 stimulation induces a unique output of IFN-β induction [31]. TLR3 binds the adaptor TICAM-1 [32, 33]. Overexpressed TICAM-1 induces IFN-β, suggesting the importance of this adaptor in selecting the IFN-β-inducing pathway (fig. 1). Ultimately, this adaptor also acts in the TLR4 IFN-β-inducing pathway [34, 35]. These results are consistent with those obtained from TRIF–/– mice [33].

IRF-3 activation induced by TLR4 signaling is independent of MyD88 and TIRAP/Mal, and instead depends on TICAM-1. To be more exact, another adaptor called TRAM (TICAM-2) serves as a bridging adaptor between TLR4 and TICAM-1 [34, 35]. TICAM-2 is similar to TICAM-1 in terms of the amino acid sequence of its Toll/IL-1 receptor homology (TIR) domain (fig. 1). In TICAM-2-deficient mice, both the MyD88-dependent pathway and the MyD88-independent pathway downstream of TLR4 signaling are defective, but TLR3 signaling remains intact [33]. TLR3 signaling also induces IRF-3 activation and IFN-β production through direct coupling with TICAM-1 [32, 34]. The IRF-3-activating pathway by TLR3/4 is greatly impaired in TICAM-1-deficient cells [36]. Thus, TICAM-1 is critically involved in TLR3 and TLR4 signaling.

Two noncanonical Iκ-B kinase homologs, i.e. Iκ-B kinase-epsilon (IKKε) (also known as inducible IKK) [36, 37] and TRAF family member-associated NF-κB activator (TANK)-binding kinase-I [TBK-I; also known as NF-κB-activating kinase (NAK)], are involved in TICAM-1-induced IRF-3 activation (fig. 1). These kinases physically couple with regulatory molecules, i.e. NAK-associated protein 1 (NAP1) or SINTBAD, and the complex can associate with TICAM-1 and induce phosphorylation and nuclear localization of IRF-3 [38, 39]. Furthermore, IRF-3-dependent gene expression induced by TLR3 and TLR4 signaling has been found to be defective in TRAF3–/– cells. Thus, TICAM-1 associates with TRAF3 for TLR3 and TLR4 signaling, causing the activation of IKKε/TBK-1 and IRF-3 [40, 41].

Signaling Pathways That Operate through the IPS-1 (MAVS, Cardif) Adaptor

Since most RNA viruses replicate in the cytoplasm, membrane proteins including TLR cannot recognize virus-replicating dsRNA in the cytoplasm. RIG-I, a member of the RNA helicase family of PRRs, has been identified with ubiquitous distribution in the cytoplasm [42]. RIG-I contains 2 caspase-recruiting domain (CARD)-like domains, i.e. DExD/H box RNA helicase and RHIM domains [42]. The helicase and RHIM domains interact with dsRNA or the 5′-triphosphate of virus-derived RNA with a short RNA duplex, whereas the CARD-like domains are required for interaction with IPS-1, an adaptor for activating downstream signaling pathways [17]. Furthermore, 2 additional RIG-I-like RNA helicases have been identified: MDA5 and LGP2 [43, 44]. MDA5 contains 2 CARD-like domains and a helicase domain, similar to RIG-I. MDA5 recognizes relatively long dsRNA in cytoplasm, whereas LGP2 lacks the CARD-like domains and is thought to positively regulate RIG-I and MDA5. These 2 signaling sensors recruit IPS-1, and IPS-1 induces the activation of IKKε/TBK-1 and IRF-3 [17] in a similar way to TICAM-1 (fig. 1).

IPS-1 contains a transmembrane domain that is rich in hydrophobic residues in its C-terminal tail and targets IPS-1 to the mitochondria [17, 45]. Notably, mitochondrial retention of IPS-1 is essential for IRF-3, IRF-7, and NF-κB activation, suggesting that signaling from mitochondria plays an important role in the antiviral immune response (fig. 1). The NS3/4A serine protease encoded by the hepatitis C virus has been demonstrated to target IPS-1 for cleavage [45, 46]. Using an in vitro cell culture infection system to introduce the hepatitis C virus, a putative cleavage site of IPS-1 was found to be located upstream of the transmembrane domain. The cleaved form of IPS-1, which lacks the transmembrane region, fails to activate IFN-β and nuclear factor (NF)-κB.

MDA5 detects long polyI:C or dsRNA, whereas RIG-I detects short dsRNA or the 5′-triphosphate end of RNA generated by viral polymerases [47, 48], indicating that these RNA helicases have different roles in the detection of viruses. Which of these pathways predominantly senses virus species is under examination. The simple interpretation that MDA5 is required for the recognition of picornaviruses and RIG-I recognizes that dsRNA-forming viruses [49] may be amended following the analysis of many virus species.

Mf, mDC, and fibroblast cells derived from RIG-I- or MDA5-deficient mice still displayed type I IFN induction in response to polyI:C stimulation, and the production of type I IFN was still observed in pDCs derived from IPS-1–/– mice [50, 51]. However, it is notable that the 2 pathways in the TLR system and the cytoplasmic IPS-1 pathway are required for dsRNA responses in different situations and cell types [50, 52]. Collectively, these observations indicate that the various modes of the RNA pattern-sensing system cooperate to detect cytoplasmic virus replication in a variety of tissues/organs.

NK Cell Activation via the TLR2/MyD88 Pathway in Mf and mDC

In vitro stimulation of mouse mDC or Mf with TLR2 stimulators BCG-CWS (cell wall skeleton) or Pam2 lipopeptide causes these cells to become NK activation inducers [24, 53]. Both TLR2 and MyD88 are indispensable for this type of NK cell activation. At least in in vitro studies, MyD88–/– Mf fail to reciprocally activate NK cells via cell-cell contact [54]. Mf MyD88 signaling through TLRs is reported to induce expression of the NKG2D ligand retinoic acid early induced transcript (RAE)-1 [55]. NK cells are then activated by MyD88-stimulated Mf. The NKG2D receptor on NK cells is downregulated by the RAE-1-NKG2D interaction [55]. MyD88 can be activated via the inflammasome pathway in human Mf [56] without the participation of TLR2. IL-1β or IL-18 (or possibly IL-33) liberated from *Plasmodium*-infected Mf may convert these cells into NK-activating Mf through IL-1β/IL-18 receptors [57, 58]. MyD88 in NK cells also participates in this type of NK activation [53, 59].

However, this is not the case in mDC. Although IL-12p70 is produced in mDC in response to the TLR2 ligand BCG-CWS, the role of IL-12 in NK activation is peripheral in the mouse system. There are at least 2 modes of MyD88-dependent NK cell activation in mDC: (1) MyD88 in pDC can couple with TLR7 or TLR9 to activate the IFN-α-inducing pathway, and this pathway also participates in NK cell activation, presumably through type I IFN liberated by pDC [60, 61]; (2) in contrast, MyD88–/– mDC lose the ability to mature in response to TLR2 ligands. For example, the TLR2 ligand Pam2CSK4 facilitates mDC-mediated NK cell activation in the case of wild-type mDC [24]. If wild-type mDC are replaced with MyD88–/– mDC, cell contact-mediated NK activation is hampered even when the cells are stimulated with Pam2 lipopeptide. Although TLR2–/– mDC largely abrogate the NK cell-activating function, slight functional activity remains in TLR2–/– mDC compared with MyD88–/– mDC, suggesting the presence of TLR2-independent MyD88-activating pathways, which may reflect the action of NOD-like receptor inflammatory pathways. Hence, NK cell activation proceeds through TLR2-dependent and TLR2-independent pathways of mDC, both of which involve MyD88. Furthermore, TLR2/MyD88 in NK cells minimally participates in direct NK activation [24, 53].

NK Cell Activation via the TICAM-1 or IPS-1 Pathways in mDC

It has long been established that a dsRNA analog, i.e. polyI:C, serves as an inducer of NK activation [62, 63]. In vivo administration of polyI:C to mice and in vitro exogenous addition of polyI:C to a mixture of BMDC and NK cells both resulted in activation of NK cells. Ex vivo studies using cells from KO mice have helped reveal how polyI:C activates NK cells. PolyI:C, unlike viral or in vitro-transcribed dsRNA, is internalized into the endosome and cytoplasm where it is recognized by TLR3 and MDA5, respectively [9, 11] (fig. 2). IPS-1 is the adaptor for MDA5/RIG-I, while TICAM-1 is the adaptor for TLR3 [32, 43, 64] (fig. 1). Using the gene-disrupted mouse cells of MDA5–/– and TLR3–/– or IPS-1–/– and TICAM-1–/– mDC, the pathway more involved in NK cell activation was examined in vitro [9–11]. In BMDC, the MDA5/IPS-1 pathway is more important than the TLR3/TICAM-1 pathway, as determined by the expression of NK activation marker CD69 and NK cytotoxicity [11], whereas the reverse is true in IFN-γ production by NK cells [64]. NK activation induced in mice injected with polyI:C is completely abrogated in double KO (IPS-1–/– and TICAM-1–/–) mice, suggesting that these 2 pathways are both required for polyI:C-mediated NK activation [10, 11].

Cell contact-mediated NK activation was found to dominantly occur in IRF-3 activation [64] and mDC by depletion studies [65]. Cytokines, including IL-12p40, IL-

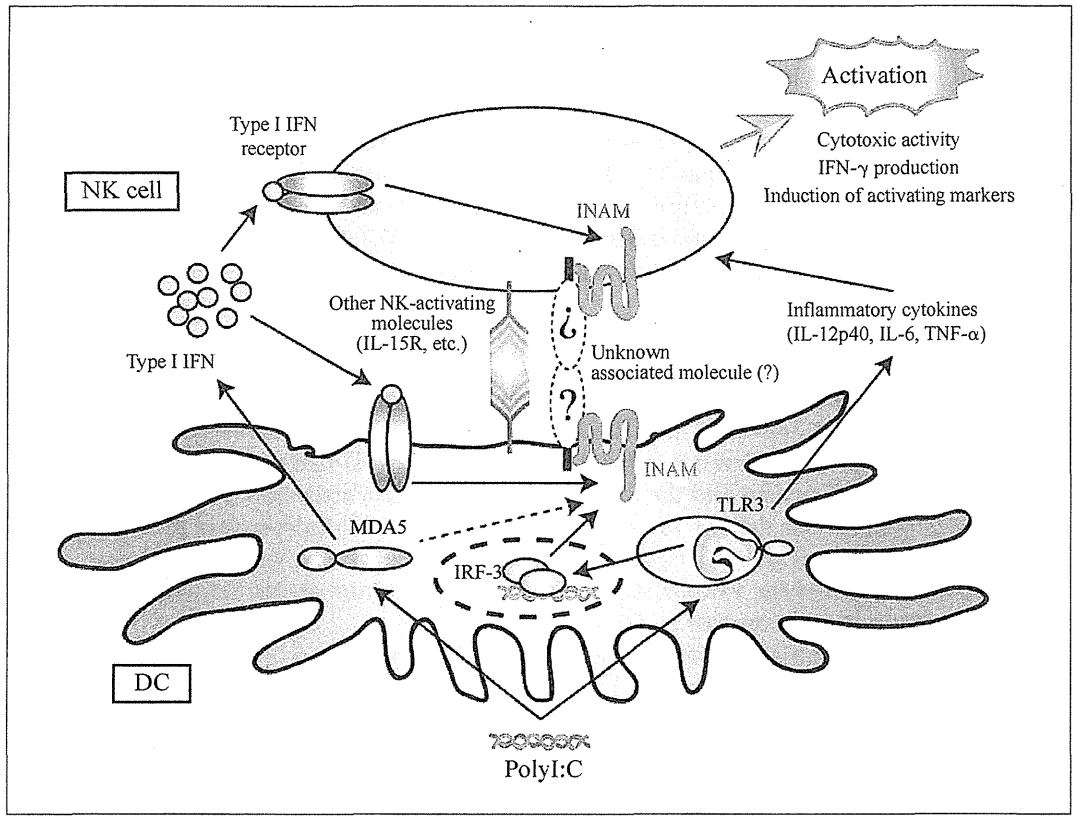

Fig. 2. Induction of INAM-mediated NK cell activation. PolyI:C-stimulated BMDC induce NK cell activation in vitro. INAM is responsible for BMDC contact-mediated NK activation. IRF-3 activation by polyI:C is crucial for INAM upregulation in BMDC, which results in the BMDC-mediated activation of NK cells. Upregulation of INAM on NK cells also facilitates NK cell activation in mDC-NK coculture.

15, and type I IFN, are produced in mDC in response to polyI:C stimulation. Both cell contact and cytokine production mediate NK activation in vitro but cytokines are known to be dispensable for NK activation by polyI:C-stimulated mDC in mice [64]. In vivo injected polyI:C allowed mice to mature splenic DCs [10, 11]. In vivo, bone marrow transplantation chimera analysis suggested that the TLR3-TICAM-1 pathway is important in myeloid cells, along with the IPS-1 pathway in nonmyeloid cells, in driving cytolytic activity by polyI:C [11] (fig. 3). Thus, nonmyeloid-derived soluble factors (mainly IFN-α/β) operate in NK activation in this case. In addition, splenic CD8α+ DC rather than CD8α– DC is crucial for driving NK activation via cell-cell contact [10].

The molecule responsible for mDC-NK contact activation has recently been investigated [64]. There are several polyI:C-inducible membrane-associated molecules in mDC and one of these molecules, designated INAM (IRF-3-dependent NK cell activating molecule), participates in mDC-NK reciprocal activation (fig. 2). However, when overexpressed in non-NK target cells, INAM does not act as an NK-activating ligand; it works only on mDC/Mf for NK activation. Since INAM is predicted to have a tetraspanin-like sequence, unidentified molecules coupling to INAM may foster mDC-NK contact.

NK Cell Activation in Humans and Mice

In human monocyte-derived DC [66] and mouse CD8α+-like human DC (BDCA3+/XCR1+) [67, 68], the early response to dsRNA (including polyI:C and polyA:U) induces the production of IL-12p40 and type I IFN via the TLR3/TICAM-1 pathway. These early-phase cytokines

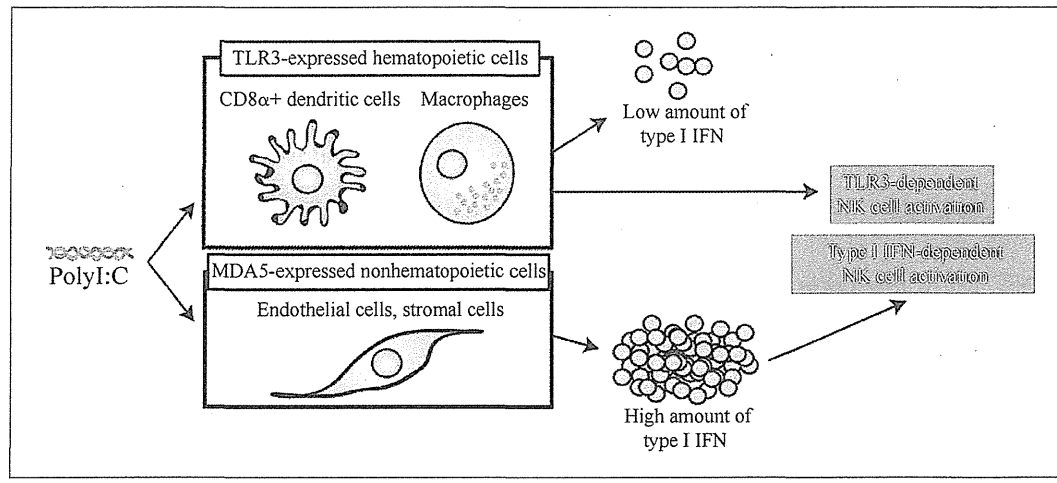

Fig. 3. The IPS-1 pathway in nonmyeloid cells and TLR3 in myeloid cells participate in the activation of NK cells. Mice i.v. injected with polyI:C activate NK cells. Two distinct pathways are involved in NK cell activation in this case: (1) TLR3 in Mf and mDC recognize the i.v. injected polyI:C and drive the internal NK activation pathway, and (2) nonhematopoietic cells recognize the polyI:C by their MDA5 in the cytoplasm to produce a large amount of type I IFN, which in turn activates Mf/DC for NK activation.

play a key role in priming NK cells to induce a low amount of IFN-γ in vitro. In vitro administered dsRNA also activates NK cells via direct stimulation of the RIG-I pathway in NK cells. In the following phase, mDC are recruited to draining lymph nodes to encounter NK cells. Subsequently, mDC-NK contact occurs inducing full maturation of NK cells. At this stage, NK cells engage in significant IFN-γ production. This recent observation is in close agreement with the results reported in human in vitro cocultured liver DC and NK cells [69]. These findings clearly reveal the importance of mDC IL-12p70 and the NK cell RIG-I pathway in NK cell priming in humans.

In mouse in vitro studies, mouse BMDC or CD8α+ splenic DC activate NK cells via cell-cell contact rather than IL-12p70 or type I IFN. BMDC, as well as CD8α+ splenic DC, express TLR3 mRNA, and polyI:C stimulation induces activation of both the TICAM-1 pathway and the IPS-1 pathway [9–11, 64]. IL-12p40 (which is likely the p40 dimer that inhibits IL-12R-derived signaling), instead of IL-12p70, is produced in response to polyI:C in mouse DC-NK coculture studies [64]. A membrane molecule, i.e. INAM, expressed secondary to IRF-3 activation in BMDC or CD8α+ DC stimulates initial DC-mediated NK activation. Full activation of NK cells, including cytolytic activity against target molecules, is provoked only secondarily to DC-NK contact in mice.

In contrast, in vivo studies on NK cell activation have been performed in mice via intravenous (i.v.) injection of polyI:C. NK activation occurs in response to i.v. injected polyI:C, and NK activation has been shown to depend on the MDA5/IPS-1 pathway and TLR3/TICAM-1pathway in KO mice [11] (fig. 3). Surprisingly, in bone marrow chimera studies, initial induction of type I IFN by MDA5 from nonmyeloid cells played a crucial role in splenic DC maturation. CD8α+ DC maturation secondarily triggered NK cell activation. If this is the case, myeloid cell TLR3 and nonmyeloid cell MDA5 actually participate in polyI:C-dependent maturation of splenic DC to drive NK activation, at least at an early phase of i.v. administration in vivo. Presumably, stromal or vascular endothelial cells are a source of MDA5-mediated type I IFN induction, which in turn activates splenic DC. There are several subsets of DC in the mouse spleen. Only CD8α+ DC express high TLR3 and participate in NK activation [10, 70]. On the other hand, intraperitoneal administration of polyI:C first activates Mf in the peritoneal cavity of mice [71]. The route of polyI:C administration may therefore stimulate different RNA sensors to activate IRF-3. IL-12p70 has a minimal role in mouse DC-mediated NK activation.

It is currently unclear which mode of NK activation, TICAM-1 or IPS-1, is dominant in other mammals and vertebrates. NK cells and the TICAM-1 and IPS-1 pathways are conserved across vertebrates. Differential re-

sponses to polyI:C in cytokine production and NK activation in other animals may be partly due to the systemic differences in RNA recognition in different cell types and tissues.

Perspectives

NK cell activation is an important event in the immune response to cancer or infectious diseases. Recent molecular/cellular analyses suggest that the cells and molecular mechanisms involved in NK cell activation differ between in vivo PAMP-stimulated mice and in vitro PAMP-stimulated cell cocultures. A discrepancy also appears in in vitro NK activation studies in humans and mice. The basal IFN-inducing properties have been reported in mice but not in humans [6]. The response to PAMP also differs depending on the delivery routes. Caution is therefore necessary when adapting the results from KO mice to human clinical studies. Only 5 μg of polyI:C sufficiently induces IFN-β from human fibroblasts which express TLR3 on the cell surface, but >150 μg is still insufficient for mouse fibroblasts [72, 73]. Although polyI:C is effective for NK activation, it often induces a life-threatening cytokine storm in mice [74]. A future aim is to activate NK cells with no side effects in human patients. If this can be achieved, NK cell activation could be applied to the establishment of effective vaccines and immunotherapies.

Acknowledgements

We are grateful to Drs. H. Oshiumi, H. Takaki, A. Watanabe, H.H. Aly, S. Yamazaki, and T. Ebihara for invaluable discussions. This work was supported in part by Grants in Aid from the Ministry of Education, Science, and Culture and the Ministry of Health, Labor, and Welfare of Japan, and by the NorthTec Foundation, the Yakult Foundation, the Waxman Foundation, and the Akiyama Life Science Foundation. Financial support from the Sapporo Biocluster 'Bio-S' the Knowledge Cluster Initiative of the MEXT, and the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT, is gratefully acknowledged.

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Rev. Med. Virol. (2011)
Published online in Wiley Online Library
(wileyonlinelibrary.com)
DOI: 10.1002/rmv.680

Reviews in Medical Virology

REVIEW

Antiviral responses induced by the TLR3 pathway

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SUMMARY

Antiviral responses are successively induced in virus-infected animals, and include primary innate immune responses such as type I interferon (IFN) and cytokine production, secondary natural killer (NK) cell responses, and final cytotoxic T lymphocyte (CTL) responses and antibody production. The endosomal Toll-like receptors (TLRs) and cytoplasmic RIG-I-like receptors (RLRs), which recognize viral nucleic acids, are responsible for virus-induced type I IFN production. RLRs are expressed in most tissues and cells and are primarily implicated in innate immune responses against various viruses through type I IFN production, whereas nucleic acid-sensing TLRs, TLRs 3, 7, 8 and 9, are expressed on the endosomal membrane of dendritic cells (DCs) and play distinct roles in antiviral immunity. TLR3 recognizes viral double-stranded RNA taken up into the endosome and serves to protect the host against viral infection by the induction of a range of responses including type I IFN production and DC-mediated activation of NK cells and CTLs, although the deteriorative role of TLR3 has also been reported in some virus infections. Here, we review the current knowledge on the role of TLR3 during viral infection, and the current understanding of the TLR3-signalling cascade that operates via the adaptor protein TICAM-1 (also called TRIF). Copyright © 2011 John Wiley & Sons, Ltd.

Received: 16 October 2010; Revised: 21 December 2010; Accepted: 21 December 2010

INTRODUCTION

Mammalian cells possess several defense strategies against viral infection, of which, the type I interferon (IFN) system is most important for innate and adaptive antiviral responses [1,2]. Type I IFN induces an antiviral state in uninfected host cells by upregulating IFN-stimulated genes (ISGs) through IFN-α/β receptor signalling, and also activates innate and adaptive immune cells, such as dendritic cells (DCs), natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) [3]. Intrinsic double-stranded RNA (dsRNA) sensors, dsRNA-binding protein kinase R and 2′–5′ oligoadenylate synthetase, are both ISGs, which trigger the shut-down of protein translation and induce RNA degradation within virus-infected cells, respectively [4,5]. Recent progressive studies have demonstrated that the endosomal Toll-like receptors (TLRs) and cytoplasmic retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) are responsible for virus-induced type I IFN production [6–8]. These receptors recognize viral nucleic acids and induce type I IFN, inflammatory cytokine and chemokine production and DC maturation. TLR3 recognizes virus-derived dsRNA and its synthetic analogue, polyriboinosinic:polyribocytidylic acid (poly (I:C)) [9–11]. dsRNA is found in some virus particles as a viral genome and can be generated

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Abbreviations:
CT, C-terminal; CTL, cytotoxic T lymphocytes; CVB3, coxsackievirus group B serotype 3; dsRNA, double-stranded RNA; DC, dendritic cell; DUBA, deubiquitinating enzyme A; ECD, ectodomain; EMCV, encephalomyocarditis virus; HCV, hepatitis C virus; HSV-1, herpes simplex virus-1; IAV, influenza A virus; IFN, interferon; INAM, IRF-3-dependent NK-activating molecule; ISG, IFN-stimulated gene; LRR, leucine-rich repeat; MCMV, murine cytomegarovirus; MDA5, melanoma differentiation associated gene 5; NAK, NF-κB activating kinase; NAP1, NAK-associated protein 1; NK, natural killer; NT, N-terminal; NTD, N-terminal domain of TICAM-1; pDC, plasmacytoid DC; poly(I:C), polyriboinosinic:polyribocytidylic acid; PVR, poliovirus receptor; RIG-I, retinoic acid inducible gene-I; RIP1, receptor-interacting protein 1; ss, single-stranded; TBK1, TANK-binding kinase 1; TICAM-1, TIR-containing adaptor molecule-1; TIR, Toll-IL-1 receptor; TLR, Toll-like receptor; RLR, RIG-I-like receptor; WNV, West Nile virus.

during the process of positive-stranded RNA virus and DNA virus replication [12]. TLR7 and TLR8 recognize virus-derived single-stranded (ss) RNA, while TLR9 recognizes non-methylated CpG-containing DNA that is found in some microbes [13–15]. Since these TLRs localize to the endosomal membranes of myeloid or plasmacytoid DCs (pDCs), they appear to detect extracellular viral nucleic acids released from infected cells or virus particles. However, the mechanism by which TLRs encounter virus-derived nucleic acids in endosomes remains to be determined. Interestingly, a recent report showed that TLR7-mediated IFN-α secretion by pDCs in response to ssRNA virus infection requires the transport of cytosolic viral RNA into the lysosome via the process of autophagy [16]. Whether this autophagy-dependent viral recognition is applicable to TLRs 3, 8 and 9 remains unclear.

By contrast, RLRs are expressed in most tissues and cells and detect viral nucleic acids in the cytoplasm. RIG-I recognizes viral RNA genomes bearing 5′-triphosphates and panhandle structures and also short-length dsRNAs [17–21], while melanoma differentiation-associated gene 5 (MDA5) detects long-length dsRNAs and poly(I:C) [22]. Studies using gene-disrupted mice and cells revealed that RIG-I is essential for the detection of various negative-stranded RNA viruses including influenza A virus (IAV), Sendai virus and vesicular stomatitis virus and a positive-stranded RNA virus, hepatitis C virus (HCV), whereas MDA5 plays a key role in sensing encephalomyocarditis virus, a member of *Picornavilridae* family [23–26]. Thus, multiple innate immune pathways are implicated in dsRNA responses and each pathway plays a distinct role in antiviral responses. In this review, we focus on TLR3, whose antiviral function has been controversial, but recent studies have demonstrated the critical role of the TLR3–TICAM-1 pathway in antiviral responses and the induction of adaptive immunity.

Expression and subcellular localization of TLR3

Human TLR3 mRNA has been detected in various tissues including the placenta, pancreas, lung, liver, heart and brain [27]. Interestingly, in the human central nervous system, TLR3 is expressed constitutively in neurons, astrocytes and microglia, suggesting a role in the response to viruses causing encephalopathy [28–30]. In immune cells, only myeloid DCs and macrophages express TLR3. The pDCs, which express TLR7 and TLR9 and secrete large amounts of IFN-α in response to viral infection, do not express TLR3 [31–35]. TLR3 is also expressed in fibroblasts and a variety of epithelial cells, including airway, corneal, cervical, biliary and intestinal cells [10,36–38], which are target sites of virus infection. TLR3 localizes both on the cell surface and endosomes in fibroblasts, macrophages and some of epithelial cell lines. Cell surface-expressed TLR3 participates in dsRNA recognition, as shown by the finding that an anti-human TLR3 monoclonal antibody (mAb) (TLR3.7) inhibits poly(I:C)-induced IFN-β production by fibroblasts [10]. By contrast, myeloid DCs only express TLR3 intracellularly [35]. Subcellular localization analysis showed that endogenous human TLR3 localizes to the early endosome but not to late endosomes/lysosomes in HeLa cells [39], while transfected human TLR3 predominantly localizes to multivesicular bodies in the mouse B-cell line Ba/F3, in which TLR3 was stably expressed at high levels. In any case, TLR3 signalling arises in the endosomal compartment, requiring endosomal maturation [35]. The 'linker' region consisting of 26 a.a. between the transmembrane domain and the Toll-IL-1 receptor (TIR) domain of TLR3, determines intracellular localization of TLR3 [40,41]. An unidentified molecule associating with the linker region may regulate the endosomal retention of TLR3 in myeloid DCs.

Notably, TLR3 expression is upregulated by viral infection and the exogenous addition of poly(I:C) or type I IFN [42]. The IFN-responsive element is located at approximately −30 bp in the human *TLR3* promoter region [43,44].

Recognition of dsRNA by TLR3

TLR3 recognizes dsRNA through its ectodomain (ECD), which induces receptor dimerization required for adaptor-mediated signal transduction [45]. TLR3 consists of an ECD formed by 23 leucine-rich repeats (LRRs) and N- and C-terminal flanking regions, known as the LRR N-terminal (LRR-NT) and C-terminal (LRR-CT) regions, the transmembrane domain and the cytoplasmic TIR domain [46] (Figure 1A). TLR3–ECD possesses 15 putative carbohydrate-binding motifs. Structural analyses

DOI: 10.1002/rmv

TICAM-1 via the RIP homotypic interaction motif domain in the C-terminal region and acts as an NF-κB inducer and apoptosis mediator in TICAM-1-mediated signalling [70–72]. TRAF6 has also been implicated in NF-κB activation by TICAM-1 in a cell-type-dependent manner [64,73].

TLR3–TICAM-1-mediated signalling is negatively regulated by a fifth TIR adaptor protein SARM [74]. SARM and TICAM-1 have been shown to interact and SARM strongly suppresses NF-κB activation, as well as IRF-3 activation by TICAM-1. Moreover, deubiquitinating enzyme A (DUBA) negatively regulates TLR3-mediated type I IFN production. DUBA selectively cleaves the Lys63-linked polyubiquitin chains on TRAF3, resulting in its dissociation from the downstream-signalling molecules [75]. In addition, the ubiquitin-modifying enzyme A20 inhibits TICAM-1-mediated NF-κB activation by deubiquitinating TRAF6 [76]. However, the precise mechanisms by which TRAF3 and TRAF6 are ubiquitinated and their interaction with downstream-signalling molecules are unknown.

Antiviral function of TLR3

The role of TLR3 in viral infection is complex (Table 1). Studies in TLR3-deficient ($TLR3^{-/-}$) mice showed that the immune response to different viruses, including lymphocytic choriomeningitis virus (an ambisense RNA virus), vesicular stomatitis virus (a negative-stranded RNA virus), murine cytomegarovirus (MCMV, a dsDNA virus) and reovirus (a dsRNA virus), was unaffected in these mutant mice compared with wild-type mice [77].

By contrast, Hardarson *et al.* [78] reported that TLR3 is important in host defense against encephalomyocarditis virus (EMCV, a positive sense ssRNA virus belonging to the *Picornaviridae* family). When mice were inoculated intraperitoneally with 50 plaque-forming units EMCV, $TLR3^{-/-}$ mice were more susceptible to EMCV infection and had a significantly high viral load in the heart compared with wild-type mice. Opposing to these data, Kato *et al.* [24] showed that MDA5 but not TLR3 plays an important role in host defense against EMCV infection, when mice were infected with 100 plaque-forming units EMCV intraperitoneally. It is unclear why these different results were obtained from similar EMCV infection studies.

Table 1. The role of TLR3 in antiviral responses

	References
Protection	
Flaviviridae [+, ss]	
West Nile virus (WNV)	[84]
Picornaviridae [+, ss]	
Encephalomyocarditis virus (EMCV)	[78]
Poliovirus	[79,80]
Coxsackievirus group B serotype 3 (CVB3)	[82]
Herpesviridae [dsDNA]	
Murine cytomegarovirus (MCMV)	[90]
Herpes simplex virus 1 (HSV-1)	[101]
Deterioration	
Flaviviridae [+, ss]	
West Nile virus (WNV)	[83]
Orthomyxoviridae [−, ss]	
Influenza A virus (IAV)	[88]
Bunyaviridae [−, ss]	
Phlebovirus	[89]

More recently, the essential role of the TLR3–TICAM-1 pathway in protection from poliovirus infection, a virus belonging to the *Picornaviridae* family, has been demonstrated [79,80]. Poliovirus receptor (PVR)-transgenic/TICAM-1-deficient mice are more susceptible than PVR-transgenic mice to intraperitoneal or intravenous inoculation with a low titre of poliovirus [79,80]. Forty-eight hours after infection, virus titres in serum dramatically increased and mortality greatly decreased compared with PVR-transgenic or PVR-transgenic/IPS-1 (RLR adaptor)-deficient mice. It is well known that in cultured mammalian cells, poliovirus infection results in inhibition of cellular protein synthesis so-called 'shut-off' event [81]. Therefore, mRNA upregulation of RIG-I and MDA5 by type I IFN does not link to protein synthesis at an early stage of virus infection. Thus,

DOI: 10.1002/rmv

it appears that the inhibitory effects of viral multiplication on host cells depend on the TLR3–TICAM-1 pathway, but not the RLR–IPS-1 pathway.

In addition, Negishi *et al.* [82] showed that TLR3$^{-/-}$ mice are more vulnerable to coxsackievirus group B serotype 3 (CVB3, a virus belonging to the *Picornaviridae* family) than wild-type mice, in terms of higher mortality and acute myocarditis. The expression of IL-12p40, IL-1β and IFN-γ mRNAs, but not IFN-β mRNA, was impaired in the hearts of CVB3-infected TLR3-deficient mice compared with those of wild-type mice infected with CVB3. By contrast, expression of TLR3 by transgene protects mice from lethal CVB3 infection and hepatitis even in the absence of type I IFN signalling. Antibody blocking studies revealed that TLR3–TICAM-1-dependent type II IFN (IFN-γ) production is critical for host defense against CVB3 infection [82].

Remarkably, Wang *et al.* [83] demonstrated that TLR3 is involved in the viral pathogenesis of West Nile virus (WNV, a positive-stranded RNA virus). TLR3$^{-/-}$ mice showed impaired cytokine production and enhanced viral loads in the periphery, whereas in the brain, the viral load, inflammatory responses and neuropathology were reduced compared with wild-type mice [83]. TLR3-mediated peripheral inflammatory cytokine production is critical for disruption of the blood–brain barrier, which facilitates viral entry into the brain causing lethal encephalitis. Therefore, TLR3$^{-/-}$ mice are more resistant to lethal WNV infection. In contrast, Daffis *et al.* [84] reported the protective role of TLR3 in sublethal WNV infection. The absence of TLR3 enhances WNV mortality in mice and increases viral burden in the brain after inoculation with the pathogenic New York strain of WNV, although there are little differences in WNV-specific antibody responses, CD8$^+$ T-cell activation, blood–brain barrier permeability and IFN-α/β induction in draining lymph nodes and serum, between wild-type and TLR3$^{-/-}$ mice [84]. The reason why TLR3 shows the opposite function against WNV infection remains to be determined.

In other RNA viral infections such as respiratory syncytial virus, IAV and phlebovirus (all negative-stranded RNA viruses), TLR3-dependent inflammatory cytokine and chemokine production also appears to affect virus-induced pathology and host survival [85–89]. TLR3$^{-/-}$ mice infected with IAV exhibited reduced inflammatory mediators, leading to increased survival [88]. It is notable that experimental conditions using high viral doses may lead to the over-production of inflammatory cytokines and chemokines. However, what type of TLR3-expressing cells that respond to virus-derived dsRNA *in vivo* has not been shown in these studies.

Cellular immunity induced by the TLR3–TICAM-1 pathway

In addition to type I IFNs, CTLs and NK cells are also principal effector cells in antiviral immunity. The contribution of TLR3 to antiviral responses has been shown in MCMV infection [90], during which virus clearance is partly dependent on NK cell activation. TLR3$^{-/-}$ mice are hypersusceptible to MCMV infection. Cytokine (type I IFN, IL-12p40 and IFN-γ) production, and NK cell and NKT cell activation are impaired in TLR3$^{-/-}$ mice compared with wild-type mice.

Selective TLR3 expression in myeloid DCs but not in pDCs raises the possibility that TLR3 also plays a key role in the antiviral response by induction of adaptive immune responses rather than primary IFN-α/β production (Table 2). Myeloid DCs are the most effective professional antigen-presenting cells, possessing several antigen processing and transporting pathways [91,92]. One of the most notable features of myeloid DCs is the cross-presentation of exogenous antigens to CD8$^+$ T cells. This pathway is important for effective host CTL induction against viruses that do not directly infect DCs. Among the myeloid DC subsets, the splenic CD8α$^+$ DC subset in mice and the CD141(BDCA3)$^+$DNGR-1(CLEC9A)$^+$ DC subset in humans highly express TLR3 and display a superior capacity for cross-presenting apoptotic and necrotic cell antigens after TLR3 stimulation [93–97]. Using TLR3-deficient mice, Schultz *et al.* [98] clearly showed that TLR3 plays an important role in cross-priming. Mouse CD8α$^+$ DCs are activated by phagocytosis of apoptotic bodies from virally infected cells or cells containing poly(I:C) in a TLR3-dependent manner. Furthermore, immunization with virally infected cells or cells containing poly(I:C), both carrying ovalbumin antigen, induces ovalbumin-specific CD8$^+$ T-cell responses, which are largely dependent on TLR3-expressing DCs [98]. In many cases, virally infected cells produce IFN-α/β which activates DCs to

DOI: 10.1002/rmv