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-

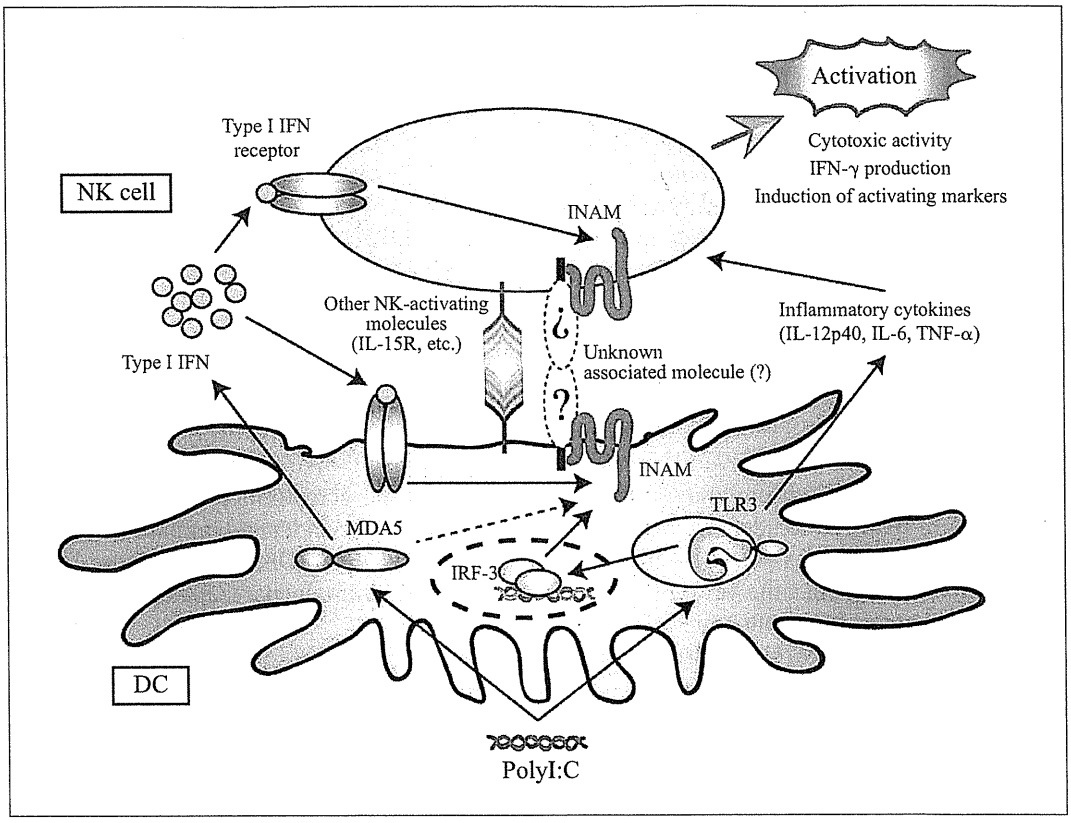

Fig. 2. Induction of INAM-mediated NK cell activation. PolyI:C-stimulated BMDC induce NK cell activation in vitro. INAM is responsible for BMDC contact-mediated NK activation. IRF-3 activation by polyI:C is crucial for INAM upregulation in BMDC, which results in the BMDC-mediated activation of NK cells.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

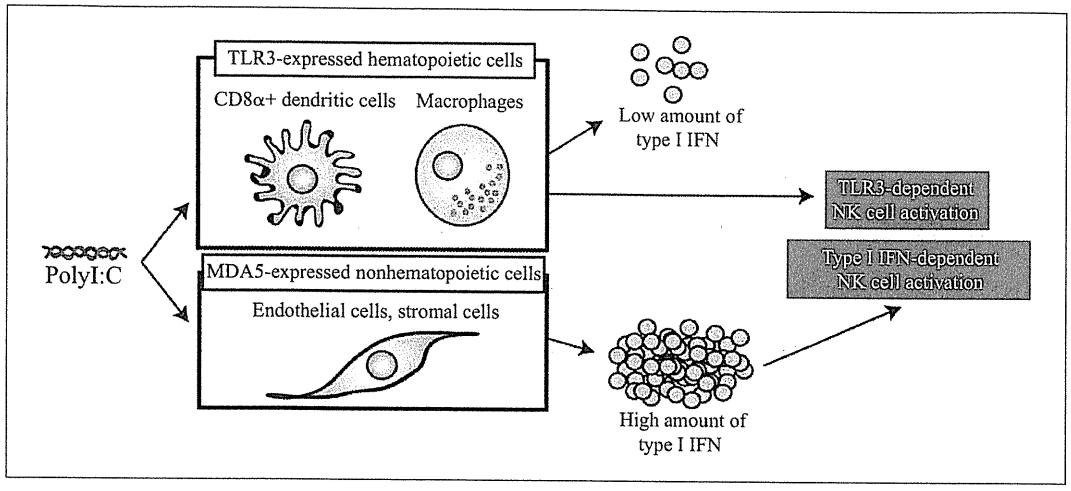

Fig. 3. The IPS-1 pathway in nonmyeloid cells and TLR3 in myeloid cells participate in the activation of NK cells. Mice i.v. injected with polyI:C activate NK cells. Two distinct pathways are involved in NK cell activation in this case: (1) TLR3 in Mf and mDC recognize the i.v. injected polyI:C and drive 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.

References

1 Trinchieri G: Biology of natural killer cells. Adv Immunol 1989;47:187–376.
2 Bryceson YT, March ME, Ljunggren HG, Long EO: Activation, coactivation, and costimulation of resting human natural killer cells. Immunol Rev 2006;214:73–91.
3 Moretta A: Natural killer cells and dendritic cells: rendezvous in abused tissue. Nat Rev Immunol 2002;2:957–964.
4 Granucci F, Zanoni I, Ricciardi-Castagnoli P: Central role of dendritic cells in the regulation and deregulation of immune responses. Cell Mol Life Sci 2008;65:1683–1697.
5 Joffre O, Nolte MA, Spörri R, Reis e Sousa C: Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. Immunol Rev 2009;227:234–247.
6 Takaoka A, Taniguchi T: New aspects of IFN-α/β signalling in immunity, oncogenesis and bone metabolism. Cancer Sci 2003; 94:405–411.
7 Kono H, Rock KL: How dying cells alert the immune system to danger. Nat Rev Immunol 2008;8:279–289.
8 Seya T, Shime H, Ebihara T, Oshiumi H, Matsumoto M: Pattern recognition receptors of innate immunity and their application to tumor immunotherapy. Cancer Sci 2010;101:313–320.
9 Akazawa T, Ebihara T, Okuno M, Okuda Y, Shingai M, Tsujimura K, Takahashi T, Ikawa M, Okabe M, Inoue N, Okamoto-Tanaka M, Ishizaki H, Miyoshi J, Matsumoto M, Seya T: Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. Proc Natl Acad Sci USA 2007;104:252–257.
10 Miyake T, Kumagai Y, Kato H, Guo Z, Matsushita K, Satoh T, Kawagoe T, Kumar H, Jang MH, Kawai T, Tani T, Takeuchi O, Akira S: Poly I:C-induced activation of NK cells by CD8 alpha+ dendritic cells via the IPS-1 and TRIF-dependent pathways. J Immunol 2009;183:2522–2528.
11 McCartney S, Vermi W, Gilfillan S, Cella M, Murphy TL, Schreiber RD, Murphy KM, Colonna M: Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. J Exp Med 2009;206:2967–2976.
12 Lanier LL: Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol 2008;9:495–502.
13 Dimasi N, Moretta L, Biassoni R: Structure of the Ly49 family of natural killer (NK) cell receptors and their interaction with MHC class I molecules. Immunol Res 2004;30:95–104.
14 Sun JC, Beilke JN, Lanier LL: Adaptive immune features of natural killer cells. Nature 2009;457:557–561.
15 Cooper MA, Colonna M, Yokoyama WM: Hidden talents of natural killers: NK cells in innate and adaptive immunity. EMBO Rep 2009;10:1103–1110.
16 Newman KC, Riley EM: Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. Nat Rev Immunol 2007;7:279–291.
17 Kawai T, Akira S: The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol 2009;21:317–337.
18 Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL: Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science 2002;296:1323–1326.
19 Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y, Strominger JL, Yewdell JW, Porgador A: Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human natural killer cells. Nature 2001;409:1055–1060.
20 Bieback K, Lien E, Klagge IM, Avota E, Schneider-Schaulies J, Duprex WP, Wagner H, Kirschning CJ, ter Meulen V, Schneider-Schaulies S: Hemagglutinin protein of wild-Type measles virus activates Toll-like receptor 2 signaling. J Virol 2002;76:8729–8736.

21 Uehori J, Fukase K, Akazawa T, Uematsu T, Akira S, Funami S, Shingai M, Matsumoto M, Azuma I, Toyoshima K, Kusumoto S, Seya T: Dendritic cell maturation induced by muramyl dipeptide (MDP) derivatives: monoacylated MDP confers TLR2/TLR4 activation. J Immunol 2005;174:7096–7103.
22 Korbel DS, Newman KC, Almeida CR, Davis DM, Riley EM: Heterogenous human NK cell responses to *Plasmodium falciparum*-infected erythrocytes. J Immunol 2005;175:7466–7473.
23 Becker I, Salaiza N, Aguirre M, Delgado J, Carrillo-Carrasco N, Kobeh LG, Ruiz A, Cervantes R, Torres AP, Cabrera N, González A, Maldonado C, Isibasi A: Leishmania lipophosphoglycan (LPG) activates NK cells through Toll-like receptor-2. Mol Biochem Parasitol 2003;130:65–74.
24 Azuma M, Sawahata R, Akao Y, Ebihara T, Yamazaki S, Matsumoto M, Hashimoto M, Fukase K, Fujimoto Y, Seya T: The peptide sequence of diacyl lipopeptides determines dendritic cell TLR2-mediated NK activation. PLoS One 2010;5:e12550.
25 Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S: Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity 1998;9:143–150.
26 Kawai T, Adachi O, Ogawa T, Takeda K, Akira S: Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 1999;11:115–122.
27 Honda K, Ohba Y, Yanai H, Negishi H, Mizutani T, Takaoka A, Taya C, Taniguchi T: Spatiotemporal regulation of MyD88-IRF-7 signaling for robust type I interferon induction. Nature 2005;434:1035–1040.
28 Uematsu S, Akira S: Toll-like receptors and type I interferons. J Biol Chem 2007;282:15319–1532.
29 Honda K, Taniguchi T: IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol 2006;6:644–658.
30 Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, Kano S, Honda K, Ohba Y, Mak TW, Taniguchi T: Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature 2005;434:243–249.
31 Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, Yamamoto A, Seya T: Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol 2003;171:3154–3162.
32 Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T: TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat Immunol 2003;4:161–167.
33 Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S: Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003;301:640–643.
34 Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, Seya T: TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. J Biol Chem 2003;278:49751–49762.
35 Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, Monks B, Pitha PM, Golenbock DT: LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the Toll adapters TRAM and TRIF. J Exp Med 2003;198:1043–1055.
36 Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM, Maniatis T: IKK-epsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 2003;4:491–496.
37 Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J: Triggering the interferon antiviral response through an IKK-related pathway. Science 2003;300:1148–1151.
38 Sasai M, Oshiumi H, Matsumoto M, Inoue N, Fujita F, Nakanishi M, Seya T: Cutting edge: NF-kappaB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. J Immunol 2005;174:27–30.
39 Ryzhakov G, Randow F: SINTBAD, a novel component of innate antiviral immunity, shares a TBK1-binding domain with NAP1 and TANK. EMBO J 2007;26:3180–3190.
40 Häcker H, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG, Kamps MP, Raz E, Wagner H, Häcker G, Mann M, Karin M: Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature 2006;439:204–207.
41 Oganesyan G, Saha SK, Guo B, He JQ, Shahangian A, Zarnegar B, Perry A, Cheng G: Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. Nature 2006;439:208–211.
42 Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T: The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 2004;5:730–737.
43 Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale M Jr, Akira S, Yonehara S, Kato A, Fujita T: Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J Immunol 2005;175:2851–2858.
44 Saito T, Hirai R, Loo YM, Owen D, Johnson CL, Sinha SC, Akira S, Fujita T, Gale M Jr: Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. Proc Natl Acad Sci USA 2007;104:582–587.
45 Li XD, Sun L, Seth RB, Pineda G, Chen ZJ: Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc Natl Acad Sci USA 2005;102:17717–17722.
46 Loo YM, Owen DM, Li K, Erickson AK, Johnson CL, Fish PM, Carney DS, Wang T, Ishida H, Yoneyama M, Fujita T, Saito T, Lee WM, Hagedorn CH, Lau DT, Weinman SA, Lemon SM, Gale MJ: Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. Proc Natl Acad Sci USA 2006;103:6001–6006.
47 Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G: 5′-Triphosphate RNA is the ligand for RIG-I. Science 2006;314:994–997.
48 Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, Weber F, Reis e Sousa C: RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. Science 2006;314:997–1001.
49 Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S: Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 2006;441:101–105.
50 Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S: Cell type-specific involvement of RIG-I in antiviral response. Immunity 2005;23:19–28.
51 Kochs G, Bauer S, Vogt C, Frenz T, Tschopp J, Kalinke U, Waibler Z: Thogoto virus infection induces sustained type I interferon responses that depend on RIG-I-like helicase signaling of conventional dendritic cells. J Virol 2010;84:12344–12350.
52 Matsumoto M, Oshiumi H, Seya T: Antiviral responses induced by the TLR3 pathway. Rev Med Virol 2011, Epub ahead of print.
53 Martinez J, Huang X, Yang Y: Direct TLR2 signaling is critical for NK cell activation and function in response to vaccinia viral infection. PLoS Pathog 2010;6:e1000811.
54 Sawaki J, Tsutsui H, Hayashi N, Yasuda K, Akira S, Tanizawa T, Nakanishi K: Type 1 cytokine/chemokine production by mouse NK cells following activation of their TLR/MyD88-mediated pathways. Int Immunol 2007;19:311–320.
55 Hamerman JA, Ogasawara K, Lanier LL: Cutting edge: Toll-like receptor signaling in macrophages induces ligands for the NKG2D receptor. J Immunol 2004;17:2001–2005.

56 Cole LE, Santiago A, Barry E, Kang TJ, Shirey KA, Roberts ZJ, Elkins KL, Cross AS, Vogel SN: Macrophage proinflammatory response to *Francisella tularensis* live vaccine strain requires coordination of multiple signaling pathways. J Immunol 2008;180:6885–6891.
57 Adachi K, Tsutsui H, Kashiwamura S, Seki E, Nakano H, Takeuchi O, Takeda K, Okumura K, Van Kaer L, Okamura H, Akira S, Nakanishi K: *Plasmodium berghei* infection in mice induces liver injury by an IL-12- and Toll-like receptor/myeloid differentiation factor 88-dependent mechanism. J Immunol 2001;167:5928–5934.
58 Baratin M, Roetynck S, Lépolard C, Falk C, Sawadogo S, Uematsu S, Akira S, Ryffel B, Tiraby JG, Alexopoulou L, Kirschning CJ, Gysin J, Vivier E, Ugolini S: Natural killer cell and macrophage cooperation in MyD88-dependent innate responses to *Plasmodium falciparum*. Proc Natl Acad Sci USA 2005; 102:14747–14752.
59 Akao Y, Ebihara T, Masuda H, Saeki Y, Akazawa T, Hazeki K, Hazeki O, Matsumoto M, Seya T: Enhancement of antitumor natural killer cell activation by orally administered Spirulina extract in mice. Cancer Sci 2009; 100:1494–501.
60 Krug A, French AR, Barchet W, Fischer JA, Dzionek A, Pingel JT, Orihuela MM, Akira S, Yokoyama WM, Colonna M: TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. Immunity 2004;21:107–119.
61 Hoshino K, Sugiyama T, Matsumoto M, Tanaka T, Saito M, Hemmi H, Ohara O, Akira S, Kaisho T: IkappaB kinase-alpha is critical for interferon-alpha production induced by Toll-like receptors 7 and 9. Nature 2006; 440:949–953.
62 Djeu JY, Heinbaugh JA, Holden HT, Herberman RB: Augmentation of mouse natural killer cell activity by interferon and interferon inducers. J Immunol 1979;122: 175–181.
63 Wiltrout RH, Salup RR, Twilley TA, Talmadge JE: Immunomodulation of natural killer activity by polyribonucleotides. J Biol Response Mod 1985;4:512–517.
64 Ebihara T, Azuma M, Oshiumi H, Kasamatsu J, Iwabuchi K, Matsumoto K, Saito T, Taniguchi T, Matsumoto M, Seya T: Identification of a polyI:C-inducible membrane protein that participates in dendritic cell-mediated natural killer cell activation. J Exp Med 2010;207:2675–2687.
65 Lucas M, Schachterle W, Oberle K, Aichele P, Diefenbach A: Dendritic cells prime natural killer cells by trans-presenting interleukin 15. Immunity 2007;26:503–517.
66 Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, Yamamoto A, Seya T: Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol 2003;171:3154–3162.
67 Crozat K, Guiton R, Contreras V, Feuillet V, Dutertre CA, Ventre E, Vu Manh TP, Baranek T, Storset AK, Marvel J, Boudinot P, Hosmalin A, Schwartz-Cornil I, Dalod M: The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells. J Exp Med 2010;207:1283–1292.
68 Bachem A, Güttler S, Hartung E, Ebstein F, Schaefer M, Tannert A, Salama A, Movassaghi K, Opitz C, Mages HW, Henn V, Kloetzel PM, Gurka S, Kroczek RA: Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. J Exp Med 2010;207:1273–1281.
69 Tu Z, Bozorgzadeh A, Pierce RH, Kurtis J, Crispe IN, Orloff MS: TLR-dependent cross talk between human Kupffer cells and NK cells. J Exp Med 2008;205:233–244.
70 Jelinek I, Leonard JN, Price GE, Brown KN, Meyer-Manlapat A, Goldsmith PK, Wang Y, Venzon D, Epstein SL, Segal DM: TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection. J Immunol 2011;186:2422–2429.
71 Wu CY, Monie A, Yang HY, Wu Tc, Hung CF: Intraperitoneal administration of polyI:C with polyethylenimine leads to significant antitumor immunity against murine ovarian tumors. Cancer Immunol Immunother 2011, in press.
72 Matsumoto M, Kikkawa S, Kohase M, Miyake K, Seya T: Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. Biochem Biophys Res Commun 2002;293:1364–1369.
73 Matsumoto M, Seya T: TLR3: interferon induction by double-stranded RNA including poly(I:C). Adv Drug Deliv Rev 2008;60:805–812.
74 Absher M, Stinebring WR: Toxic properties of a synthetic double-stranded RNA: endotoxin-like properties of poly I – poly C, an interferon stimulator. Nature 1969;223:715–717.

Rev. Med. Virol. 2011; **21**: 67–77.
Published online 10 February 2011 in Wiley Online Library
(wileyonlinelibrary.com)
DOI: 10.1002/rmv.680

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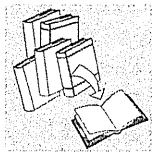
REVIEW



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

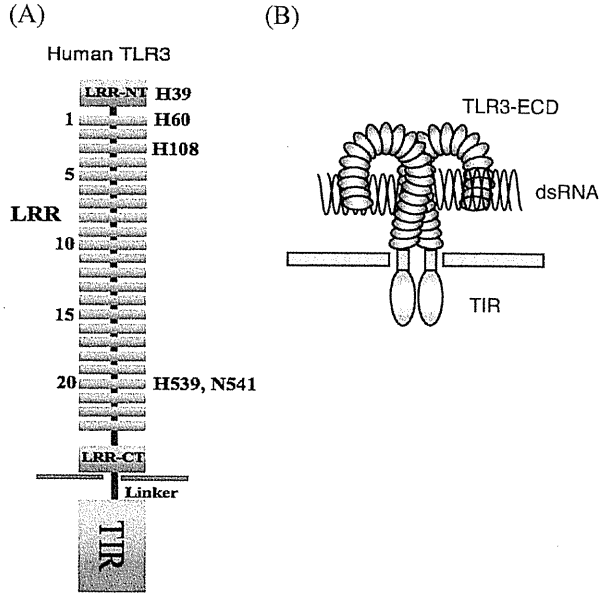

Figure 1. (A) Schematic structure of human TLR3. TLR3 is a type I transmembrane protein of 904 a.a. TLR3 consists of an ECD formed by 23 LRRs and N- and C-terminal flanking regions (LRR-NT and LRR-CT), the transmembrane domain, cytoplasmic linker region and the TIR domain. H539 and N541 in TLR3-LRR20, H39 in the LRR-NT, H60 in LRR1 and H108 in LRR3 are essential for dsRNA-binding. (B) Model of the dsRNA–TLR3-signalling complex. dsRNA interacts with both an N- and a C-terminal binding site on the glycan-free surface of each TLR3–ECD, which are located on opposite sides of the dsRNA (53).

of human TLR3–ECD revealed that the LRRs form a large horseshoe-shaped solenoid of which one face is largely masked by carbohydrate, while the other face is unglycosylated [47,48]. By point mutation analysis, Bell *et al.* [49] demonstrated that the His539 and Asn541 residues in TLR3–LRR20, located on the glycan-free lateral face, are critical amino acids for dsRNA binding and signalling. Wild-type TLR3–ECD protein directly binds poly(I:C) at pH7.6, while mutant proteins H539E and N541A fail to bind poly(I:C). Based on the observation that an acidic pH (pH 6.0 and below) is required for TLR3 recognition of dsRNA, the N-terminal conserved histidine residues, His39 in the LRR-NT, His60 in LRR1 and His108 in LRR3, were identified as a second binding site for dsRNA [50,51]. Protonation of these imidazole groups under acidic conditions, such as those found in endosomes, appears to generate an ionic interaction between the histidine residues and the negatively charged phosphate backbone of dsRNA.

In addition, Leonard *et al.* [52], showed that TLR3–ECD binds as a dimer to 40–50 bp length of dsRNA, and multiple TLR3–ECD dimers bind to long dsRNA strands. Binding affinities increase with both buffer acidity and dsRNA length. At the pH within early endosomes (~6.0–6.5), >90-bp length of dsRNA is required to form a stable complex with TLR3. However, at the pH within late endosomes (~5.5 and below), 40–50-bp length of dsRNA forms stable complex with dimeric TLR3, suggesting that dsRNA-induced TLR3-mediated signalling depends on the length of the dsRNA and the TLR3 localization site [52]. Finally, structural analysis of the complex of two mouse TLR3–ECDs and one 46-bp dsRNA oligonucleotide revealed that dsRNA interacts with both an N- and a C-terminal binding site on the glycan-free surface of each mTLR3–ECD, which are located on opposite sides of the dsRNA [53] (Figure 1B). The dsRNA in the complex retains a typical A-form DNA-like structure. dsRNA has been predicted to adopt a right-handed A-form helix with 11 bp per helical turn and a 28 Å helical pitch [54]. Therefore, two helical turns would fit between the N- and C-terminal binding sites of TLR3 [53]. In addition, the two LRR-CT domains are brought into proximity and form a series of protein–protein interactions, which facilitate the dimerization of the cytoplasmic TIR domain. Funami *et al.* [40], reported that the Phe732, Leu742 and Gly743 residues in the TLR3 cytoplasmic linker region are essential for TLR3 signalling, suggesting that the linker region controls the dimerization of the TLR3–TIR domain.

TLR3–TICAM-1-signalling pathway

TLR3 mediates signalling via an adaptor protein, TIR-containing adaptor molecule-1 (TICAM-1; also called TRIF) [55,56] (Figure 2A). TICAM-1 activates the transcription factors IRF-3, NF-κB and AP-1, leading to the induction of type I IFN, cytokine/chemokine production and DC maturation, which then enables the activation of NK cells and CTLs. TLR3 also associates with c-Src tyrosine kinase on endosomes in response to dsRNA [57]. The Src kinase inhibitor markedly inhibits dsRNA-elicited phosphorylation of Akt, a downstream target of phosphatidylinositol 3-kinase (PI3-K). In addition, PI3-K is required for full phosphorylation and activation of IRF-3 by dsRNA [58]. The precise role of c-Src in IRF-3 activation via the PI3-K-Akt pathway requires further elucidation.

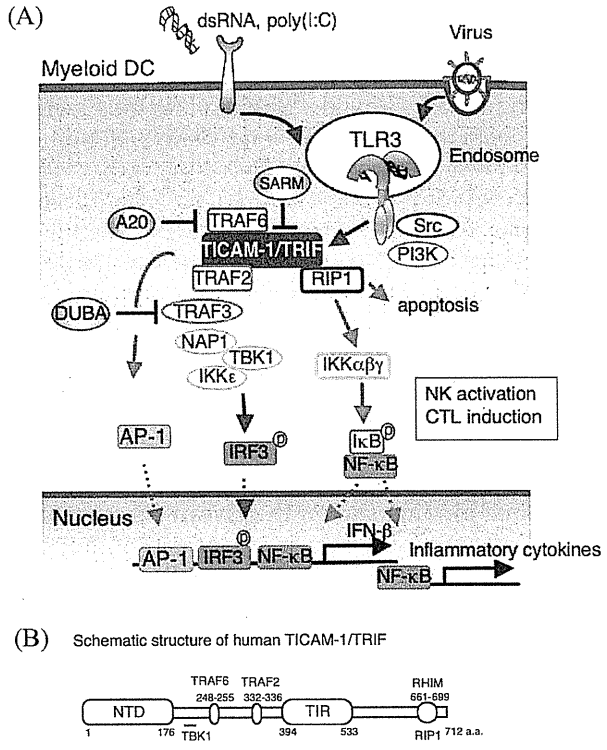

Figure 2. (A) TLR3–TICAM-1-signalling pathway. In myeloid DCs, TLR3 is expressed in the endosomal compartments and recognizes extracellular viral dsRNA and its synthetic analogue poly(I:C). Once TLR3 is dimerized by dsRNA, it recruits the adaptor protein TICAM-1/TRIF that activates the transcription factors, IRF3, NF-κB and AP-1. RIP1 associates with TICAM-1 via the PHIM domain in the C-terminal region and acts as an NF-κB activator and apoptosis mediator in TICAM-1-mediated signalling. TRAF3 and NAP1 participate in the recruitment and activation of the IRF-3 kinases TBK1 and IKKε. Phosphorylated IRF-3 translocates into the nucleus and together with NF-κB and AP-1 induces IFN-β gene transcription. The TICAM-1-mediated AP-1 activation pathway is unclear. (B) Schematic structure of human TICAM-1/TRIF. N-terminal domain (NTD) (1–176 a.a.), TIR domain (394–533 a.a.), RHIM domain (661–699 a.a.), TRAF6-binding site (248–256 a.a.), TRAF2-binding site (332–336 a.a.) and TBK1-binding site (under line) are shown.

TICAM-1 consists of an N-terminal region, a TIR domain and a C-terminal region (Figure 2B). The TIR domain of TICAM-1 is essential for binding to the TIR domain of TLR3 and also to the TLR4 adaptor TICAM-2 (also called TRIF-related adaptor molecule) [59,60]. TICAM-1 is expressed at a low level in most tissues and cells and is diffusely localized in the cytoplasm of resting cells [39]. When endosomal TLR3 is activated by dsRNA, TICAM-1 transiently co-localizes with TLR3, then dissociates from the receptor and forms speckled structures that co-localize with downstream-signalling molecules [39]. Homo-oligomerization through the Pro434 residue in the TIR domain and the C-terminal region is essential for TICAM-1-mediated activation of NF-κB and IRF-3 [61]. Once TICAM-1 is oligomerized, the serine-threonine kinases, TANK-binding kinase 1 (TBK1; also called NAK or T2K) and IkB kinase-related kinase-ε (IKK-ε; also called IKK-i), are activated and phosphorylate IRF-3 [62,63]. The ubiquitin ligase of the TRAF family members, TRAF2, TRAF3 and TRAF6, are downstream-signalling molecules of TICAM-1. TRAF2 and TRAF6 directly bind to the N-terminal region of TICAM-1 [64,65] (Figure 2B). The Lys63-linked autoubiquitination of TRAF3 is required for IRF-3 activation [66,67]. Furthermore, NF-κB-activating kinase (NAK)-associated protein 1 (NAP1) participates in the recruitment of IRF-3 kinases to the N-terminal region of TICAM-1 [68]. Although both TRAF3 and NAP1 associate with oligomerized TICAM-1 and serve as a critical link between TICAM-1 and downstream IRF-3 kinases, there is no evidence that they bind directly to TICAM-1. Interestingly, recent reports showed that direct binding of TBK1 to TICAM-1 is necessary for IRF-3 activation [69]. The Leu194 residue in the N-terminal region is critical for TBK1 binding to TICAM-1. In addition, the Ser189, Arg195 and Ser196 residues are involved in TBK1–TICAM-1 binding.

The N-terminal 176 a.a. of TICAM-1 form a protease-resistant structural domain, designated NTD (Figure 2B). Because the crucial amino acids for TRAF2-, TRAF6- and TBK1-binding reside between the NTD and the TIR domain, naive TICAM-1 may have a closed conformation that covers these binding sites. Indeed, protein–protein interaction analysis revealed that the NTD interacts with the N-terminus of TICAM-1–TIR [69]. Thus, the NTD folds into the TIR domain structure to maintain the naive conformation of TICAM-1. Upon stimulation of TLR3 or TLR4, TICAM-1 oligomerizes through the TIR domain and the C-terminal region, possibly breaking the intramolecular association and inducing a conformational change that allows TBK1 access to TICAM-1.

Whereas the N-terminal region is crucial for TICAM-1-mediated IRF-3 activation, the C-terminal region of TICAM-1 is involved in NF-κB activation and apoptosis. Receptor-interacting protein 1 (RIP1), a kinase containing a death domain, associates with

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,

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

Table 2. Expression of nucleic acid-sensing TLRs in DC subsets

	DC subset	TLR3	TLR7	TLR8	TLR9	References
Human	Myeloid DC					
	MoDC	+	−	+	−	[31–35]
	$CD11c^+CD1c^+$DC	+	−	+	−	[34,35,94,95]
	$CD141^+CLEC9A^+$DC	++	−	+	−	[94,95]
	Plasmacytoid DC	−	+	−	+	[34,35]
Mouse	Myeloid DC					
	BMDC	+	−	−	+	[95]
	$CD8\alpha^+$ DC	++	−	−	+	[93,95]
	Plasmacytoid DC	−	+	−	+	[93,95]

MoDC, monocyte-derived immature dendritic cells; BMDC, bone marrow-derived DC.

promote $CD8^+$ T-cell cross-priming [99]. Thus, both TLR3- and IFN-α/β-mediated signalling are likely implicated in licensing DCs for the cross-priming of $CD8^+$ T cells.

In humans, Ebihara *et al.* [100] demonstrated the role of TLR3, expressed in myeloid DCs, in the immune response to HCV infection. The JFH1 strain of HCV does not directly infect or stimulate myeloid DCs to activate T cells and NK cells, but instead the phagocytosis of HCV-infected apoptotic cells that contain HCV-derived dsRNA and their interaction with the TLR3 pathway in myeloid DCs, plays a critical role in DC maturation and activation of T and NK cells [100]. In addition, Jongbloed *et al.* [94] reported that $CD141^+$ DCs are able to cross-present viral antigens from human cytomegalovirus-infected necrotic fibroblasts. Physiologically, TLR3 in a DC subset specialized for antigen presentation appears to encounter viral dsRNAs in the endosome by uptake of apoptotic or necrotic virus-infected cells and signals for cross-presentation of viral antigens. Furthermore, a dominant-negative TLR3 allele was found in children with herpes simplex virus 1 (HSV-1) encephalitis [101]. TLR3 is expressed in the central nervous system, where it is required to control HSV-1. Interestingly, recent paper demonstrated that mouse $CD8\alpha^+$ DCs and human $CD141^+$ DCs are major producers of IFN-λ in response to poly(I:C), which depends on TLR3 [102]. Thus, TLR3 plays a role in the antiviral response, dependent on the viral genome structure, the route of virus entry into cells, the TLR3-expressing cell type that encounters viral dsRNA, and the properties of the host anti-viral effector functions.

Application of the TLR3 ligand to adjuvant vaccine therapy

Selective expression of TLR3 in myeloid DCs, especially human $CD141^+$ DCs and mouse $CD8\alpha^+$ DC subsets, is the advantage in employing TLR3 ligands as adjuvant. In addition to the TLR3-dependent CTL activation described above, DC-mediated NK cell activation is also important for the adjuvancy of TLR3 ligands. Akazawa *et al.* [103] showed that the TLR3–TICAM-1 pathway is essential for poly(I:C)-induced NK-cell-mediated tumour regression in a syngeneic mouse tumour implant model. Remarkably, production of IFN-α is not impaired in TICAM-$1^{-/-}$ mice compared with wild-type mice after *in vivo* poly(I:C) injection or *in vitro* bone marrow-derived DC (BMDC) stimulation, whereas IL-12 production is completely dependent on TICAM-1, consistent with other reports [22,104]. Furthermore, NK cell activation requires cell–cell contact with BMDCs preactivated by poly(I:C) but not IFN-α or IL-12. Thus, the TLR3–TICAM-1 pathway in myeloid DCs facilitates the DC–NK cell interaction following NK cell activation. TICAM-1–IRF3-dependent expression of a novel molecule, namely IRF-3-dependent NK-activating molecule (INAM), in myeloid DCs is required for NK activation [104]. Poly(I:C)-induced MDA5-dependent myeloid DC activation is also implicated in NK cell activation [105,106].

However, several issues remain unresolved including a suitable transport system for TLR3 ligands. Poly(I:C) injected intraperitoneally in mice activates both TLR3 and MDA5, indicating that extracellular poly(I:C) is delivered to endosomal TLR3 and further to cytosolic MDA5 in murine cells. A recent study demonstrated that CD14 enhances poly(I:C)-mediated TLR3 activation in bone marrow-derived macrophages by directly binding to poly(I:C) and mediating cellular uptake of poly(I:C) [107]. The internalized poly(I:C) then colocalizes with CD14 and TLR3. Since the extracellular domain of CD14 consists of LRRs [108], CD14 may associate with TLR3 and transfer poly(I:C) to TLR3 in macrophage endosomes. In the case of CD14-negative myeloid DCs, extracellular dsRNA must be internalized with the putative uptake receptor. Indeed, it has been demonstrated by our group and others that poly(I:C) is internalized into human monocyte-derived immature DCs and mouse BMDCs via clathrin-dependent endocytosis, and B- and C-type oligodeoxynucleotides share the uptake receptor with poly(I:C) [109]. Notably, among various synthetic dsRNAs, poly(I:C) is preferentially internalized and activates TLR3 in myeloid DCs. By contrast, *in vitro*-transcribed dsRNAs of various lengths (50–1000 bp) cannot be internalized into myeloid DCs [110]. Thus, uptake of TLR3 ligands largely depends on the dsRNA structure recognized by the uptake receptor expressed on myeloid DCs.

The dsRNA structure and the targeting approach of dsRNA to the endosomal TLR3 in the appropriate DC subset, are important factors involved in generating innate and adaptive immune responses by TLR3 ligands. Gowen *et al.* [111] showed that poly(I:C_{12}U) induces IFN-β in a TLR3-dependent and MDA5-independent manner, and exhibits protective anti-viral effects in mice. Identification of the putative dsRNA uptake receptor is crucial for analysing the intracellular transport of dsRNA. Furthermore, clarification of the differences between the RIG-I/MDA5-mediated and TLR3–TICAM-1-mediated signalling pathways is important for assessment of dsRNA-induced immune responses.

Concluding remarks

The protective role of TLR3 in virus infection is now becoming clear from experiments using an infectious mouse model or TLR3-transgenic mice. Since both CVB3 and poliovirus belong to the *Picornaviridae* family, along with encephalomyocarditis virus that is recognized by MDA5, distinct virus properties rather than virus genome structure appear to determine which RNA sensors act in antiviral defense in host cells. The molecular mechanism behind the anti-viral function of TLR3 *in vivo* and the identification of TLR3–TICAM-1-mediated signalling cascades distinct from those of RIG-I/MDA5, are important factors for understanding the role of RNA-sensors in the host defense system. In addition, characterization of a new myeloid DC subset that expresses a high level of TLR3 and has a high capacity to present antigen from apoptotic and necrotic cells after TLR3 activation, may provide insight into the role of TLR3 in the activation of NK cells and CTLs in viral infection. This, in turn, may advance the development of TLR3-related vaccine adjuvants effective against tumours and/or infectious diseases.

REFERENCES

1. Muller U, Steinhoff U, Reis LFL, *et al.* Functional role of type I and type II interferons in antiviral defense. *Science* 1994; **264**: 1918–1921.
2. Vilcek J. Fifty years of interferon research: aiming at a moving target. *Immunity* 2006; **25**: 343–348.
3. Garcia-Sastre A, Biron CA. Type I interferons and the virus-host relationship: a lesson in Détente. *Science* 2006; **312**: 879–882.
4. Clemens MJ. PKR-a protein kinase regulated by double-stranded RNA. *International Journal of Biochemistry and Cell Biology* 1997; **29**: 945–949.
5. Samuel CE. Antiviral actions of interferon, interferon-regulated cellular proteins and their surprisingly selective antiviral activities. *Virology* 1991; **183**: 1–11.
6. Yoneyama M, Kikuchi M, Natsukawa T, *et al.* The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nature Immunology* 2004; **5**: 730–737.
7. Yoneyama M, Kikuchi M, Matsumoto K, *et al.* Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *Journal of Immunology* 2005; **175**: 2851–2858.
8. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; **124**: 783–801.
9. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. RNA and activation of NF-κB by Toll-like receptor 3. *Nature* 2001; **413**: 732–738.
10. Matsumoto M, Kikkawa S, Kohase M, Miyake K, Seya T. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Bio-*

chemical and Biophysical Research Communications 2002; **239**: 1364–1369.
11. Matsumoto M, Seya T. TLR3: interferon induction by double-stranded RNA including poly(I:C). *Advanced Drug Delivery Reviews* 2008; **60**: 805–812.
12. Weber F, Wagner V, Rasmussen SB, Hartmann R, Paludan SR. Double-stranded RNA is produced by positive-stranded RNA viruses and DNA viruses but not in detectable amounts by negative-stranded RNA viruses. *Journal of Virology* 2006; **80**: 5059–5064.
13. Hemmi H, Takeuchi O, Kawai T, *et al.* A Toll-like receptor recognizes bacterial DNA. *Nature* 2000; **408**: 740–745.
14. Heil F, Hemmi H, Hochrein H, *et al.* Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 2004; **303**: 1526–1529.
15. Diebold SS, Kaisho T, Hemmi H, Akira S, Sousa RC. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 2004; **303**: 1529–1531.
16. Lee HK, Lund JM, Ramanathan B, Mizushima N, Iwasaki A. Autophagy-dependent viral recognition by plasmacytoid dendritic cells. *Science* 2007; **315**: 1398–1401.
17. Hornung V, Ellegast J, Kim S, *et al.* 5′-Triphosphate RNA is ligand for RIG-I. *Science* 2006; **314**: 994–997.
18. Pichlmair A, Schulz O, Tan CP, *et al.* RIG-I-mediated anti-viral responses to single-stranded RNA bearing 5′-phosphates. *Science* 2006; **314**: 997–1001.
19. Schlee M, Roth A, Hornung V, *et al.* Recognition of 5′ triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* 2009; **31**: 25–34.
20. Schmidt A, Schwerd T, Hamm W, *et al.* 5′-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proceedings of the National Academy of Science of the United States of America* 2009; **106**: 12067–12072.
21. Rehwinkel J, Tan CP, Goubau D, *et al.* RIG-I detects viral genomic RNA during negative-strand RNA virus infection. *Cell* 2010; **140**: 397–408.
22. Kato H, Takeuchi O, Mikamo-Satoh E, *et al.* Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *Journal of Experimental Medicine* 2008; **205**: 1601–1610.
23. Kato H, Sato S, Yoneyama M, *et al.* Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 2005; **23**: 19–28.
24. Kato H, Takeuchi O, Sato S, *et al.* Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 2006; **441**: 101–105.
25. Gitlin L, Barchet W, Gilfillan S, *et al.* Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proceedings of the National Academy of Science of the United States of America* 2006; **103**: 8459–8464.
26. Saito T, Owen DM, Jiang F, Marcotrigiano J, Gale M Jr. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. *Nature* 2008; **454**: 523–527.
27. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JFA. A family of human receptors structurally related to *Drosophila* Toll. *Proceedings of the National Academy of Science of the United States of America* ; **95**: 588–593.
28. Prehaud C, Megret F, Lafage M, Lafon M. Virus infection switches TLR-3-positive human neurons to become strong producers of beta interferon. *Journal of Virology* 2005; **79**: 12893–12904.
29. Farina C, Krumbholz M, Giese T, Hartmann G, Aloisi F, Meinl E. Preferential expression and function of Toll-like receptor 3 in human astrocytes. *Journal of Neuroimmunology* 2005; **159**: 12–19.
30. Town T, Jeng D, Alexopoulou L, Tan J, Flavell RA. Microglia recognize double-stranded RNA via TLR3. *Journal of Immunology* 2006; **176**: 3804–3812.
31. Muzio M, Bosisio D, Polentarutti N, *et al.* Differential expression and regulation of Toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *Journal of Immunology* 2000; **64**: 5998–6004.
32. Visintin A, Mazzoni A, Spitzer JH, Wyllie DH, Dower SK, Segal DM. Regulation of Toll-like receptors in human monocytes and dendritic cells. *Journal of Immunology* 2001; **166**: 249–254.
33. Kadowaki M, Ho S, Antonenko S, *et al.* Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *Journal of Experimental Medicine* 2001; **194**: 863–870.
34. Hornung V, Rothenfusser S, Britisch S, *et al.* Quantitative expression of Toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *Journal of Immunology* 2002; **168**: 4531–4537.
35. Matsumoto M, Funami K, Tanabe M, *et al.* Subcellular localization of Toll-like receptor 3 in human dendritic cells. *Journal of Immunology* 2003; **171**: 3154–3162.
36. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of Toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infection and Immunity* 2000; **68**: 7010–7017.
37. Niimi K, Asano Y, Shiraishi T, *et al.* TLR3-mediated synthesis and release of eotaxin-1/CCL11 from human bronchial smooth muscle cells stimulated with double-stranded RNA. *Journal of Immunology* 2007; **178**: 489–495.
38. Nakamura M, Funami K, Komori A, *et al.* Increased expression of Toll-like receptor 3 in intrahepatic bilinary epithelial cells at sites of ductular reaction in diseased livers. *Hepatology International* 2008; **2**: 222–230.
39. Funami K, Sasai M, Ohba Y, Oshiumi H, Seya T, Matsumoto M. Spatiotemporal mobilization of Toll-IL-1 receptor domain-containing adaptor molecule 1 in response to dsRNA. *Journal of Immunology* 2007; **179**: 6867–6872.
40. Funami K, Matsumoto M, Oshiumi H, Akazawa T, Yamamoto A, Seya T. The cytoplasmic 'linker region' in Toll-like receptor 3 controls receptor localization and signaling. *International Immunology* 2004; **16**: 1143–1154.
41. Nishiya T, Kajita E, Miwa S, DeFranco A. TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. *Journal of Biological Chemistry* 2005; **280**: 37107–37117.
42. Miettinen M, Sareneva T, Julkunen I, Matikainen S. IFNs activate toll-like receptor gene expression in viral infections. *Genes and Immunity* 2001; **2**: 349–355.
43. Heinz S, Haehnel V, Karaghiosoff M, *et al.* Species-specific regulation of Toll-like receptor 3 genes in men and mice. *Journal of Biological Chemistry* 2003; **24**: 21502–21509.
44. Tanabe M, Taniguchi M, Takeuchi K, *et al.* Mechanism of up-regulation of human Toll-like receptor (TLR) 3 secondary to infection of measles virus attenuated strains. *Biochemical and Biophysical Research Communications* 2003; **311**: 39–48.
45. Gay NJ, Gangloff M, Weber ANR. Toll-like receptors as molecular switches. *Nature Reviews. Immunology* 2006; **6**: 693–698.

46. Bell JK, Mullen GED, Leifer CA, Mazzoni A, Davies DR, Segal DM. Leucine-rich repeats and pathogen recognition in Toll-like receptors. *Trends in Immunology* 2003; **24**: 528–533.
47. Choe J, Kelker MS, Wilson IA. Crystal structure of human Toll-like receptor 3 (TLR3) ectodomain. *Science* 2005; **309**: 581–585.
48. Bell JK, Botos I, Hall PR, *et al.* The molecular structure of the Toll-like receptor 3 ligand-binding domain. *Proceedings of the National Academy of Science of the United States of America* 2005; **102**: 10976–10980.
49. Bell JK, Askins J, Hall PR, Davies DR, Segal DM. The dsRNA binding site of human Toll-like receptor 3. *Proceedings of the National Academy of Science of the United States of America* 2006; **103**: 8792–8797.
50. Fukuda K, Watanabe T, Tokisue T, *et al.* Modulation of double-stranded RNA recognition by the N-terminal histidine-rich region of the human Toll-like receptor 3. *Journal of Biological Chemistry* 2008; **283**: 22787–22794.
51. Pirher N, Ivicak K, Pohar J, Bencina M, Jerala R. A second binding site for double-stranded RNA in TLR3 and consequences for interferon activation. *Nature Structural and Molecular Biology* 2008; **15**: 761–763.
52. Leonard JN, Ghirlando R, Askins J, *et al.* The TLR3 signaling complex forms by cooperative receptor dimerization. *Proceedings of the National Academy of Science of the United States of America* 2008; **105**: 258–263.
53. Liu L, Botos I, Wang Y, *et al.* Structural basis of Toll-like receptor 3 signaling with double-stranded RNA. *Science* 2008; **320**: 379–381.
54. Rana TM. Illuminating the silence: understanding the structure and function of small RNAs. *Nature Reviews. Molecular Cell Biology* 2007; **8**: 23–36.
55. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-β induction. *Nature Immunology* 2003; **4**: 161–167.
56. Yamamoto M, Sato S, Hemmi H, *et al.* Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. *Science* 2003; **301**: 640–643.
57. Johnsen IB, Nguyen TT, Ringdal M, *et al.* Toll-like receptor 3 associates with c-Src tyrosine kinase on endosomes to initiate antiviral signaling. *EMBO Journal* 2006; **25**: 3335–3346.
58. Sarker SN, Peters K, Elco CP, Sakamoto S, Pal S, Sen GC. Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. *Nature Structural and Molecular Biology* 2004; **11**: 1060–1067.
59. Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, Seya T. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to Toll-like receptor 4 TICAM-1 that induces interferon-β. *Journal of Biological Chemistry* 2003; **278**: 49751–49762.
60. Fitzgerald KA, Rowe DC, Barnes BJ, *et al.* LPS-TLR4 signaling to IRF-3/7 and NF-κB involves the toll adapters TRAM and TRIF. *Journal of Experimental Medicine* 2003; **198**: 1043–1055.
61. Funami K, Sasai M, Oshiumi H, Seya T, Matsumoto M. Homo-oligomerization is essential for Toll/IL-1 receptor domain-containing adaptor molecule-1 mediated NF-κB and IRF-3 activation. *Journal of Biological Chemistry* 2008; **283**: 18283–18291.
62. Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. *Science* 2003; **300**: 1148–1151.
63. Fitzgerald KA, McWhirter SM, Faia KL, *et al.* IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nature Immunology* 2003; **4**: 491–496.
64. Sato S, Sugiyama M, Yamamoto M, *et al.* Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β (TRIF) associates with TNFR-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-κB and IFN-regulatory factor 3, in the Toll-like receptor signaling. *Journal of Immunology* 2003; **171**: 4304–4310.
65. Sasai M, Tatematsu M, Oshiumi H, *et al.* Direct binding of TRAF2 and TRAF6 to TICAM-1/TRIF adaptor participates in activation of the Toll-like receptor 3/4 pathway. *Molecular Immunology* 2010; **47**: 1283–1291.
66. Hacker H, Redecke V, Blagoev B, *et al.* Specificity in Toll-like receptor signaling through distinct effector functions of TRAF3 and TRAF6. *Nature* 2006; **439**: 204–207.
67. Oganesyan G, Saha SK, Guo B, *et al.* Critical role of TRAF3 in the Toll-like receptor-dependent and –independent antiviral response. *Nature* 2006; **439**: 208–211.
68. Sasai M, Oshiumi H, Matsumoto M, *et al.* Cutting edge: NF-kappaB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain- containing adapter molecule-1-mediated IFN regulatory factor 3 activation. *Journal of Immunology* 2005; **174**: 27–30.
69. Tatematsu M, Ishii A, Oshiumi H, *et al.* A molecular mechanism for Toll/IL-1 receptor domain-containing adaptor molecule-1-mediated IRF-3 activation. *Journal of Biological Chemistry* 2010; **285**: 20128–20136.
70. Meylan E, Burns K, Hofmann K, *et al.* RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. *Nature Immunology* 2004; **5**: 503–507.
71. Han KJ, Su X, Xu LG, Bin LH, Zhang J, Shu HB. Mechanisms of the TRIF-induced interferon-stimulated response element and NF-kappaB activation and apoptosis pathways. *Journal of Biological Chemistry* 2004; **279**: 15652–15661.
72. Kaiser WJ, Offermann MK. Apoptosis induced by the Toll-like receptor adaptor TRIF is dependent on its receptor interacting protein homotypic interaction motif. *Journal of Immunology* 2005; **174**: 4942–4952.
73. Gohda J, Matsumura T, Inoue J. Cutting edge: TNFR-associated factor (TRAF) 6 is essential for MyD88-dependent pathway but not Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β (TRIF)-dependent pathway in TLR signaling. *Journal of Immunology* 2004; **173**: 2913–2917.
74. Carty M, Goodbody R, Schroder M, Stack J, Moynagh PN, Bowie A. The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling. *Nature Immunology* 2006; **7**: 1074–1081.
75. Kayagaki N, Phung Q, Chan S, *et al.* DUBA: a deubiquitinase that regulates type I interferon production. *Science* 2007; **318**: 1628–1632.
76. Boone DL, Turer EE, Lee EG, *et al.* The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses. *Nature Immunology* 2004; **5**: 1052–1060.
77. Edelman KH, Richardson-Burns S, Alexopoulou L, Tyer KL, Flavell RA, Oldstone MBA. Does Toll-like receptor 3 play a biological role in virus infections? *Virology* 2004; **322**: 231–238.
78. Hardarson HS, Baker JS, Yang Z, *et al.* Toll-like receptor 3 is an essential component of the innate stress response in virus-induced cardiac injury. *American Journal of Physiology. Heart and Circulatory Physiology* 2007; **292**: 251–258.

79. Abe Y, Nagata N, Sata T, Takeuchi O, Akira S, Koike S. TLR3-TRIF pathway is important for type I IFN responses in poliovirus infection. *Proceedings of 57th Annual Meeting of the Japanese Society for Virology* 2009; 234.
80. Oshiumi H, Matsumoto M, Seya T. TICAM-1/TRIF, a TLR3 adaptor, is essential for protection against poliovirus infection. *International Immunology* 2010 **22**(Suppl. 1) i19. (abstract).
81. Racaniello VR. Picornaviridae: the viruses and their replication. In *Fields Virology*, 5th edn, Knipe DM, Howly PM (eds). Lippincott Williams & Wilkins: Philadelphia, 2007; 795–838.
82. Negishi H, Osawa T, Ogami K, *et al.* A critical link between Toll-like receptor 3 and type II interferon signaling pathways in antiviral innate immunity. *Proceedings of the National Academy of Science of the United States of America* 2008; **105**: 20446–20451.
83. Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nature Medicine* 2004; **10**: 1366–1373.
84. Daffis S, Samuel MA, Suthar MS, Gale M Jr, Diamond MS. Toll-like receptor 3 has a protective role against West Nile virus infection. *Journal of Virology* 2008; **82**: 10349–10358.
85. Guillot L, Goffic RL, Bloch S, *et al.* Involvement o Toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *Journal of Biological Chemistry* 2005; **280**: 5571–5580.
86. Rudd BD, Burstein E, Duckett CS, Li X, Lukacs NW. Differential role for TLR3 in respiratory syncytial virus-induced chemokine expression. *Journal of Virology* 2005; **79**: 3350–3357.
87. Rudd BD, Smit JJ, Flavell RA, *et al.* Deletion of TLR3 alters the pulmonary immune environment and mucs production during respiratory syncytial virus infection. *Journal of Immunology* 2006; **176**: 1937–1942 .
88. Goffic RL, Baalloy V, Lagranderie M, *et al.* Detrimental contribution of the Toll-like receptor (TLR) 3 to influenza A virus-induced acute pneumonia. *PLoS Pathogens* 2006; **2**: 526–535.
89. Gowen BB, Hoopes JD, Wong M-H, *et al.* TLR3 deletion limits mortality and disease severity due to phlebovirus infection. *Journal of Immunology* 2006; **177**: 6301–6307.
90. Tabeta K, Georgel P, Janssen E, *et al.* Toll-like receptor 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. *Proceedings of the National Academy of Science of the United States of America* 2004; **101**: 3516–3521.
91. Heath WR, Belz GT, Behrens GM, *et al.* Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunology Reviews* 2004; **199**: 9–26.
92. Shen L, Lock KL. Priming of T cells by exogenous antigen cross-presented on MHC class I molecules. *Current Opinion in Immunology* 2006; **18**: 85–91.
93. Edwards AD, Diebold SS, Slack EM, *et al.* Toll-like receptor expression in murine DC subsets: lack of CD8 α+ DC correlates with unresponsiveness to imidazoquinolines. *European Journal of Immunology* 2003; **33**: 827–833.
94. Jongbloed SL, Kassianos AJ, McDonald KJ, *et al.* Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *Journal of Experimental Medicine* 2010; **207**: 1247–1260.
95. Poulin LF, Salio M, Griessinger E, *et al.* Characterization of human DNGR-1+ BDCA3+ leukocytes as putative equivalents of mouse CD8α+ dendritic cells. *Journal of Experimental Medicine* 2010; **207**: 1261–1271.
96. Bachem A, Güttler S, Hartung E, *et al.* Superior antigen cross-presentation and XCR1 expression define human CD11c+ CD141+ cells as homologues of mouse CD8+ dendritic cells. *Journal of Experimental Medicine* 2010; **207**: 1273–1281.
97. Crozat K, Guiton R, Contreras V, *et al.* The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8α+ dendritic cells. *Journal of Experimental Medicine* 2010; **207**: 1283–1292.
98. Schultz O, Diebold SS, Chen M, *et al.* Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 2005; **433**: 887–892.
99. Bon AL, Etchart N, Rossmann C, *et al.* Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. *Nature Immunology* 2003; **4**: 1009–1015.
100. Ebihara T, Shingai M, Matsumoto M, Wakita T, Seya T. Hepatitis C virus (HCV)-infected apoptotic cells extrinsically modulate dendritic cell function to activate T cells and NK cells. *Hepatology* 2008; **48**: 48–58.
101. Zhang S-Y, Jouanguy E, Ugolini S, *et al.* TLR3 deficiency in patients with Herpes Simplex encephalitis. *Science* 2007; **317**: 1522–1527.
102. Lauterbach H, Bathke B, Gilles S, *et al.* Mouse CD8α+ DCs and human BDCA3+ DCs are major producers of IFN-λ in response to poly(I:C). *Journal of Experimental Medicine* 2010; **207**: 2703–2717.
103. Akazawa T, Ebihara T, Okuno M, *et al.* Antitumor NK activation induced by the TLR3–TICAM-1 (TRIF) pathway in myeloid dendritic cells. *Proceedings of the National Academy of Science of the United States of America* 2007; **104**: 252–257.
104. Ebihara T, Azuma M, Oshiumi H, *et al.* Identification of a polyI:C-inducible membrane protein that participates in dendritic cell-mediated natural killer cell activation. *Journal of Experimental Medicine* 2010; **207**: 2675–2687.
105. Miyake T, Kumagai Y, Kato H, *et al.* Poly I:C-induced activation of NK cells by CD8 alpha+ dendritic cells via the IPS-1 and TRIF-dependent pathways. *Journal of Immunology* 2009; **183**: 2522–2528.
106. McCartney S, Vermi W, Gilfillan S, *et al.* Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. *Journal of Experimental Medicine* 2009; **206**: 2967–2976.
107. Lee HKS, Dunzendorfer K, Soldau K, Tobias PS. 2006; Double-stranded RNA-mediated TLR3 activation is enhanced by CD14. *Immunity* **24**: 153–163.
108. Kim J-I, Lee CJ, Jin MS, *et al.* Crystal structure of CD14 and its implications for lipopolysaccharide signaling. *Journal of Biological Chemistry* 2005; **280**: 11347–11351.
109. Itoh K, Watanabe A, Funami K, Seya T, Matsumoto M. The clathrin-mediated endocytic pathway participates in dsRNA-induced IFN-β production. *Journal of Immunology* 2008; **181**: 5522–5529.
110. Okahira S, Nishikawa F, Nishikawa S, Akazawa T, Seya T, Matsumoto M. Interferon-β induction through Toll-like receptor 3 depends on double-stranded RNA structure. *DNA and Cell Biology* 2005; **24**: 614–623.
111. Gowen BB, Wong M-H, Jung K-H, *et al.* TLR3 is essential for induction of protective immunity against Punta Toro virus infection by the double-stranded RNA (dsRNA), poly(I:C12U), but not poly(I:C): differential recognition of synthetic dsRNA molecules. *Journal of Immunology* 2007; **178**: 5200–5208.