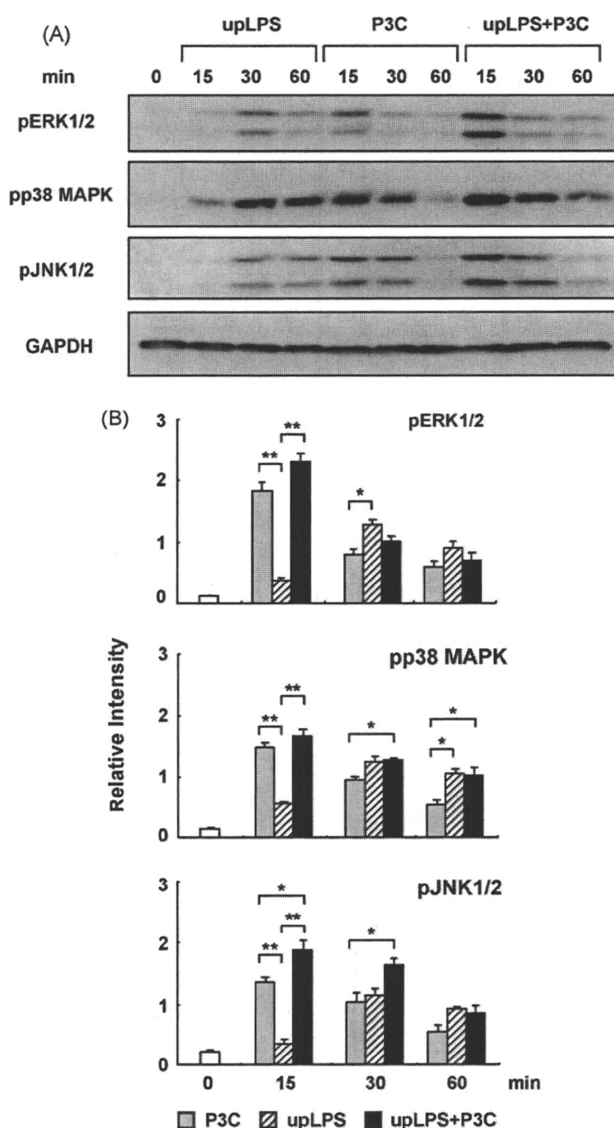

Fig. 4. Activation of ERK1/2, and p38 MAPK, and JNK1/2 in DCs upon TLR4 and/or TLR2 stimulation. BMDCs were treated with upLPS (1 μg/ml) and/or P3C (100 ng/ml) for 15, 30 or 60 min, and whole cell lysates were prepared. Levels of phospho-ERK1/2 (pERK1/2), phospho-p38 MAPK (pp38 MAPK), and phospho-JNK1/2 (pJNK) in the cell lysates were determined by immunoblotting. GAPDH level was determined as an internal control for each sample. (A) Representative immunoblot is shown. (B) The relative intensity of the specific band is shown. Each column represents the mean ± S.E. of four independent experiments. Statistical significance was calculated by one-way ANOVA with Bonferroni's post hoc test ($^{*}p < 0.05$; $^{**}p < 0.01$).

It has been reported that PI3K is involved in TLR-induced IL-10 production by murine macrophages (Pengal et al., 2006; Saegusa et al., 2007; Polumuri et al., 2007). However, we could not detect phosphorylation of Akt, a downstream effector of PI3K, in TLR4,2-stimulated DCs as well as unstimulated DCs (data not shown). Since a possibility that undetectable levels of PI3K activation might be involved in the enhanced IL-10 production by TLR4,2-stimulated DCs remained, we examined the effect of LY294002, a specific inhibitor of PI3K, on the cytokine production by DCs. LY294002 almost completely inhibited the augmented production of IL-10 by DCs stimulated with upLPS and P3C, while showing no significant effects on the IL-12 p40 production (Fig. 5).

3.6. Activation of NF-κB pathway in DCs upon TLR stimulation

TLR4/MyD88-mediated TRAF6-recruitment initiates signaling cascades that activate NF-κB pathway as well as MAPK pathway (Häcker et al., 2006; Hoebe and Beutler, 2006). It has been reported that NF-κB pathway is involved in DC production of various cytokines (Kawai and Akira, 2007). In the process of NF-κB activation, NF-κB p65 in cytoplasm is phosphorylated and subsequently translocated into the nucleus. We, thus, analyzed levels of phospho-NF-κB p65 in TLR4-, TLR2-, or TLR4,2-stimulated DCs.

BMDCs were treated with upLPS and/or P3C for indicated time periods, and intracellular proteins levels of phospho-NF-κB p65 were determined (Fig. 6). Marked phosphorylation of NF-κB p65 was observed at 15 min after P3C stimulation and the levels of phospho-NF-κB p65 were decreased at 30 and 60 min. In contrast, modest phosphorylation of NF-κB p65 was detected at 15 min after upLPS stimulation and the level of phospho-NF-κB p65 was increased at 30 and 60 min. The level of phospho-NF-κB p65 in TLR4,2-stimulated DCs was same as that in TLR2-stimulated DCs. Accordingly, the phospho-NF-κB p65 level in upLPS-stimulated DCs was lower than that in TLR2- or TLR4,2-stimulated DCs at 15 min. No significant differences were detected in the levels of phospho-NF-κB p65 between TLR4-, TLR2-, and TLR4,2-stimulated DCs at 30 and 60 min.

3.7. The role of NF-κB pathway in cytokine production by DCs upon TLR stimulation

We next examined the influence of NF-κB inhibition using PDTC, a specific inhibitor of NF-κB, on cytokine production by DCs upon TLR stimulation. BMDCs were pretreated with PDTC for 1 h and then stimulated with upLPS and/or P3C for 24 h in the presence of the inhibitor (Fig. 7). PDTC partially but significantly inhibited IL-10 production by DCs upon simultaneous stimulation with TLR4 and TLR2 ligands (Fig. 7 left). On the other hand, PDTC completely inhibited IL-12 p40 production by TLR4-, TLR2-, and TLR4,2-stimulated DCs (Fig. 7 right). This finding demonstrates that NF-κB pathway is indispensable for IL-12 p40 production by TLR4 and/or TLR2-stimulated DCs.

4. Discussion

In the innate immune system, macrophages/monocytes and DCs are activated by cell wall products of Gram-positive (Takeuchi et al., 1999; Underhill et al., 1999; Means et al., 2000) and Gram-negative bacteria (Poltorak et al., 1998; Qureshi et al., 1999), spirochetes (Brightbill et al., 1999), yeasts (Underhill et al., 1999), and mycobacteria (Underhill et al., 1999; Means et al., 1999) via TLR4 and TLR2. TLR4 binds to LPS, while TLR2 recognizes bacterial lipoproteins and peptidoglycans (Takeda et al., 2003). Following these TLR stimulation, macrophages/monocytes and DCs produce various cytokines (Trinchieri and Sher, 2007; O'Neill and Bowie, 2007). However, effects of cooperative signaling via TLR4 and TLR2 on cytokine production in DCs are not well characterized compared to those in macrophages/monocytes.

It has been reported that combinations of TLR ligands synergistically augment various cytokine production by macrophages and DCs (Napolitani et al., 2005; Bagchi et al., 2007; Theiner et al., 2008). The synergistic effect between various TLRs appears to be crucial for the innate immune defense against a variety of pathogens (Trinchieri and Sher, 2007). Most of previous studies for the TLR synergy, however, have analyzed the production of inflammatory mediators such as TNF-α and IL-12 but scarcely that of anti-inflammatory cytokines such as IL-10. In the present study, we focused on inflammatory versus anti-inflammatory cytokines production by murine conventional DCs and demonstrated that simultaneous stimulation of TLR4 and TLR2 resulted in synergistic enhancement of anti-inflammatory cytokine, IL-10, production,

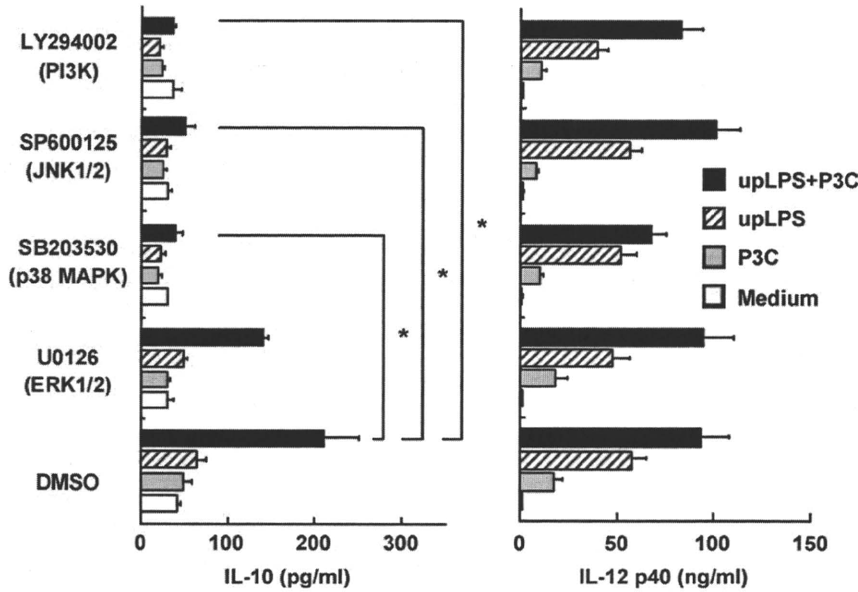

Fig. 5. IL-10 and IL-12 production by DCs upon TLR4 and/or TLR2 stimulation in the presence of ERK1/2, p38 MAPK, JNK1/2, or PI3K inhibitor. BMDCs were pretreated with 10 μM U0126 (a MEK1/2 inhibitor), 30 μM SB203530 (a p38 MAPK inhibitor), 10 μM SP600125 (a JNK1/2 inhibitor), 10 μM LY294002 (a PI3K inhibitor), or vehicle alone (0.1% DMSO) for 1 h and then stimulated with upLPS (1 μg/ml) and/or P3C (100 ng/ml) for 24 h in the presence of each inhibitor. The amount of IL-10 and IL-12 in the culture supernatants was measured by ELISA. Each column represents the mean ± S.E. of three independent experiments. Statistical significance was calculated by paired *t*-test ($^{*}p < 0.05$).

but not that of inflammatory cytokines, IL-12 p40 and TNF-α.

Sato et al. (2000) have demonstrated that mycoplasmal lipopeptides, a TLR2 ligand, acts synergistically with LPS for induction of TNF-α in murine peritoneal macrophages. However, we were unable to detect such a synergistic effect of upLPS and P3C on TNF-α production by DCs. Thus, regulation system of TLR4,2-mediated TNF-α production in DCs may be different from that in macrophages. Alternatively, it seems possible that this discrepancy is attributable to the purity (standard versus ultra-pure) and/or origin (mycoplasmal versus synthetic) of TLR4 and TLR2 ligands.

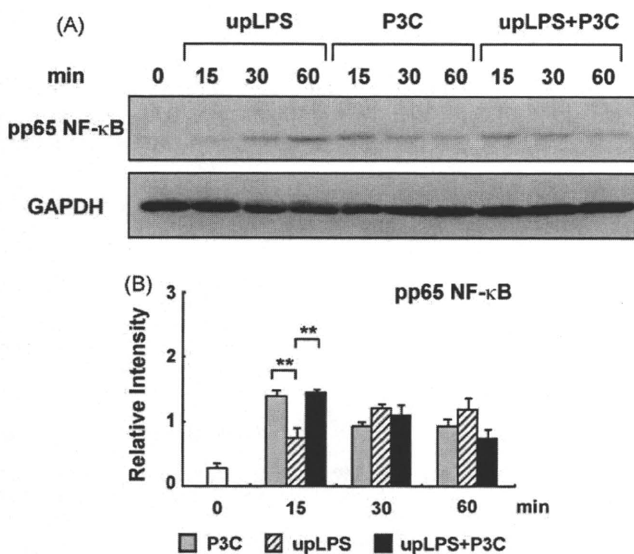

Fig. 6. Activation of NF-κB pathway in DCs upon TLR4 and/or TLR2 stimulation. BMDCs were treated with upLPS (1 μg/ml) and/or P3C (100 ng/ml) for 15, 30 or 60 min, and whole cell lysates were prepared. Levels of phospho-NF-κB p65 (pp65 NF-κB) were determined by immunoblotting. GAPDH level was determined as an internal control for each sample. (A) Representative immunoblot is shown. (B) The relative intensity of the specific band is shown. Each column represents the mean ± S.E. of four independent experiments. Statistical significance was calculated by one-way ANOVA with Bonferroni's post hoc test ($^{**}p < 0.01$).

We used two types of LPS, "standard LPS (sLPS)" and "ultra-pure LPS (upLPS)", for TLR4 stimulation. The sLPS has been generally used in most LPS stimulation studies. The sLPS failed to act with P3C in IL-10 production in a synergistic manner unlike upLPS. Our study with TLR2 knock out mice suggested that contaminated TLR2 ligands such as lipoproteins contributed to the IL-10 production by DCs upon sLPS stimulation. It seems that the contaminated TLR2 ligands in the sLPS mask the effects of additional treatment with P3C. Thus, effects of contaminated components in sLPS on DC functions observed in the previous studies should be carefully reconsidered.

Although transcription factors and signal transduction pathways for production of inflammatory cytokines have been well documented (Kawai and Akira, 2005), those for anti-inflammatory cytokines such as IL-10 have not been thoroughly characterized. It has been reported that MAPKs including ERK1/2, p38 MAPK, and JNK1/2 are involved in activation of various transcription factors that promote the IL-10 synthesis in human monocytes and murine macrophages upon LPS stimulation (Liu et al., 2006; Norkina et al., 2007). We analyzed activities of ERK1/2, p38 MAPK, and JNK1/2 in TLR4-, TLR2-, and TLR4,2-stimulated DCs in the present study. At an early phase (at 15 min), P3C activated more vigorously the ERK1/2, p38 MAPK, and JNK1/2 than upLPS. On the contrary, activities of ERK1/2 and p38 MAPK in upLPS-treated DCs were significantly higher than those in P3C-treated DCs at later phases. It seems that upLPS and P3C mainly contribute to activation of these MAPKs at an early (15 min) and later phases (30 and 60 min), respectively, after treatment of DCs with these ligands. The inhibitor study suggested that p38 MAPK and JNK1/2 were essential to induce synergistic enhancement of IL-10 production in TLR4,2-stimulated DCs. From these observations, we consider that sequential cooperation of the TLR2-mediated early activation and the TLR4-mediated late activation of MAPKs appears to be crucial for the enhanced IL-10 production by DCs upon simultaneous stimulation with TLR4 and TLR2.

PI3K is also involved in TLR-induced IL-10 production by murine macrophages (Pengal et al., 2006; Saegusa et al., 2007; Polumuri et al., 2007). Thus, we also analyzed phosphorylation of Akt, a down-

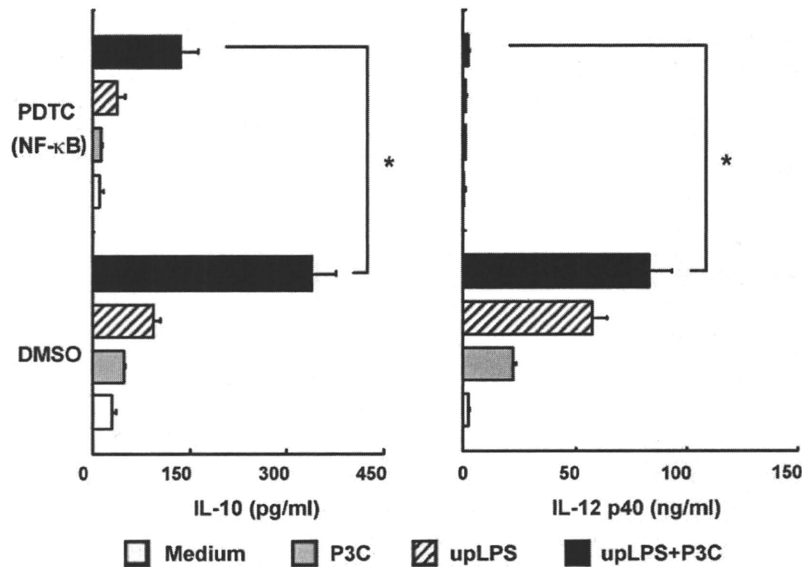

Fig. 7. IL-10 and IL-12 production by DCs upon TLR4 and/or TLR2 stimulation in the presence of NF-κB inhibitor. BMDCs were pretreated with 20 μM PDTC (an NF-κB inhibitor) for 1 h and then stimulated with upLPS (1 μg/ml) and/or P3C (100 ng/ml) for 24 h in the presence of the inhibitor. The amount of IL-10 and IL-12 in the culture supernatants was measured by ELISA. Each column represents the mean ± S.E. of three independent experiments. Statistical significance was calculated by paired *t*-test ($^{*}p < 0.05$).

stream effector of PI3K, in TLR4,2-stimulated DCs. However, we could not detect phospho-Akt in unstimulated and TLR4-, TLR2-, and TLR4,2-stimulated DCs (data not shown). On the other hand, LY294002, a specific inhibitor of PI3K, almost completely abolished the synergistic IL-10 production in TLR4,2-stimulated DCs, while showing no significant effects on the IL-12 p40 production. Undetectable level of PI3K activation may be responsible for the enhanced IL-10 production in TLR4,2-stimulated DCs.

Qian et al. (2006) showed that NF-κB pathway was involved in IL-12 production upon LPS stimulation, but was dispensable for the IL-10 production in murine BMDCs that were conditioned by co-culturing with spleen stromal cells. In contrast, Liu et al. (2006) demonstrated that NF-κB pathway was indispensable for LPS-induced IL-10 synthesis in murine macrophage cell line RAW264.7. Thus, the role of NF-κB pathway in IL-10 production upon TLR stimulation remains elusive. In the present study, we also examined the role of NF-κB pathway in the enhanced IL-10 production by DCs upon TLR4 and TLR2 stimulation. The level of NF-κB activation in TLR4,2-stimulated DCs was higher than that in TLR4 alone-stimulated DCs at an early phase (15 min). However, this difference in the NF-κB activation seen between the TLR4- and TLR4,2-stimulated DCs in the NF-κB activation was modest compared to that in MAPK activation. On the other hand, no significant differences were observed in the NF-κB activation between TLR4-, TLR2-, and TLR4,2-stimulated DCs at the later phase. Thus, it seems that the simultaneous stimulation with TLR4 and TLR2 ligands exerts a relatively slight influence on the NF-κB activation in DCs compared to that on MAPK activation. In addition, the synergy in IL-10 production by DCs upon TLR4 and TLR2 stimulation was only partially inhibited by PDTC. From these observations, it seems to us that NF-κB pathway is not as much essential for the synergy in IL-10 production by DCs upon TLR4 and TLR2 stimulation as p38 MAPK and JNK1/2 pathways are.

We have previously demonstrated that DCs primed with TLR4 and TLR2 ligands and rested for 48 h showed enhanced IL-10 production but markedly diminished IL-12 p40 production upon TLR4 restimulation (Yanagawa and Onoé, 2007). However, mechanism underlying the enhanced IL-10 and diminished IL-12 p40 production remains unclear. In this previous study (Yanagawa and Onoé, 2007), we have not analyzed cytokine production by DCs during the TLR4,2 priming (first stimulation). Our present findings indicate that DCs produced a substantial amount of IL-10 during the TLR4,2 priming. The abundant IL-10 environment during the priming might affect the DC ability to produce IL-10 and IL-12 p40 upon the second stimulation with TLR4 ligands.

In summary, we demonstrated that cooperate signaling via TLR4 and TLR2 induced the synergy in DCs production of anti-inflammatory cytokine, IL-10, but not inflammatory cytokines, IL-12 and TNF-α. Since the balance between anti-inflammatory versus inflammatory cytokine is crucial for the regulation of immune homeostasis, elucidation of the complex pathways that induce the selective synergy in IL-10 production may lead to the development of clinical applications exploiting this new regulation system for the treatment of various infectious diseases and immune disorders.

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The Clathrin-Mediated Endocytic Pathway Participates in dsRNA-Induced IFN-β Production[1]

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TLR3 and cytoplasmic RIG-I-like receptor (RLR) recognize virus-derived dsRNA and induce type I IFN production in a distinct manner. Human TLR3 localizes to the endosomal compartments in myeloid dendritic cells (mDCs), while it localizes to both the cell surface and interior in fibroblasts and epithelial cells. TLR3 signaling arises in the intracellular compartment in both cell types and requires endosomal maturation. The mechanisms by which extracellular dsRNA is delivered to the TLR3-containing organelle remain largely unknown. Among various synthetic dsRNAs, poly(I:C) is preferentially internalized and activates TLR3 in mDCs. In vitro transcribed dsRNAs hardly induce IFN-β production in mDCs. In this study, we demonstrate that the clathrin-dependent endocytic pathway mediates cell entry of poly(I:C) to induce IFN-β gene transcription. Furthermore, poly(I:C)-induced IFN-β production is inhibited by pretreatment of cells with B- and C-type oligodeoxynucleotides (ODNs) but not with TLR7/8 ligands. The binding and internalization of B-type ODNs by mDCs was reduced in the presence of poly(I:C), suggesting that poly(I:C) shares the uptake receptor with B- and C-type ODNs. Hence, foreign dsRNA is recognized by differently categorized receptors, cytoplasmic RIG-I-like receptor, membrane-bound TLR3 and cell-surface RNA capture. The endocytic pathway is critical for dsRNA-induced TLR3-mediated cell activation. *The Journal of Immunology,* **2008, 181: 5522–5529.**

Type I IFNs (IFN-α/β) play essential roles in both innate and adaptive antiviral immune responses (1, 2). Many types of cells such as fibroblasts, epithelial cells, and dendritic cells (DCs)[5] produce IFN-β upon viral infection or stimulation with poly(I:C), a synthetic analog of viral dsRNA (3). Membrane-bound TLR3 and cytoplasmic DEAD/H box RNA helicases, such as retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5, participate in the recognition of virus-derived dsRNA and induction of IFN-α/β gene transcription (4–9).

Human TLR3 localizes to the endosomal compartments in myeloid DCs (mDCs), while it localizes to both the cell-surface and interior of fibroblasts and epithelial cells (5, 10, 11). Anti-human TLR3 mAb inhibits poly(I:C)-induced IFN-β production in fibroblasts, indicating that TLR3 present on the cell surface participates in dsRNA recognition (5). However, in both cell types, TLR3 signaling arises in an intracellular compartment and requires endosomal maturation (10, 11). After dsRNA recognition, TLR3 homodimerizes, and this is followed by recruitment of an adaptor molecule, i.e., Toll-IL-1 receptor domain-containing adaptor molecule-1 (TICAM-1, also called Toll-IL-1 receptor domain-containing adaptor inducing IFN-β). This activates the NF-κB and interferon regulatory factor-3 transcription factors leading to IFN-β production (12–15). However, the mechanism by which extracellular dsRNA is delivered to the TLR3-positive organelle is unknown.

A recent study has shown that CD14 directly binds to poly(I:C) and mediates poly(I:C) cellular uptake (16). Bone marrow-derived macrophages from CD14$^{-/-}$ mice exhibited impaired responses to poly(I:C) (16). CD14 is a well-known cell-surface pattern-recognition receptor that is involved in both LPS-mediated TLR4 signaling and in TLR2 signaling (17, 18). However, mDCs do not express CD14 (19), suggesting that other cell-surface molecules mediate the entry of dsRNA into mDCs. In this study, we used pharmacological inhibitors to analyze the mechanisms by which extracellular dsRNAs activate endosomal TLR3. We found that the clathrin-dependent endocytic pathway participates in poly(I:C)-induced IFN-β production in mDCs. Furthermore, an inhibition study with various nucleic acids revealed that poly(I:C) shares its uptake receptor with B- and C-type oligodeoxynucleotides (ODNs).

Materials and Methods

Cell culture and reagents

A human embryonic kidney cell line HEK293 was obtained from Sumitomo Pharmaceuticals and maintained in DMEM supplemented with 10% heat-inactivated FCS (JRH Biosciences) and antibiotics. The HEK293 cells have no TLR3. We prepared TLR3-expressing HEK293 cells by transient transfection of the expression plasmid for human TLR3, which predominantly express TLR3 intracellularly but possess some TLR3 molecules on the cell-surface. Chloroquine, chlorpromazine, cytochalasin D, methyl-β-cyclodextrin, 4′,6-diamidino-2-phenylindole (DAPI), propidium iodide, control ODN2006, LPS from *Escherichia coli* (serotype 0111:B4), and polymyxin B were purchased from Sigma-Aldrich. Alexa Fluor 488-acetylated low-density lipoprotein (AcLDL) and Alexa Fluor 488-cholera toxin B subunit (CTXB) were from Molecular Probes. Poly(I:C) was

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[5] Abbreviations used in this paper: DC, dendritic cell; mDC, myeloid DC; TICAM-1, Toll-IL-1 receptor-containing adaptor molecule-1; ODN, oligodeoxynucleotide; AcLDL, acetylated low density lipoprotein; CTXB, cholera toxin subunit B; MV, measles virus; CIAP, calf intestine alkaline phosphatase; iDC, immature DC; pDC, plasmacytoid DC; CpG-B, B-type CpG ODN; CpG-A, a-type CpG ODN; DPBS, Dulbecco's PBS; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate; DAPI, 4′,6-diamidino-2-phenylindole.

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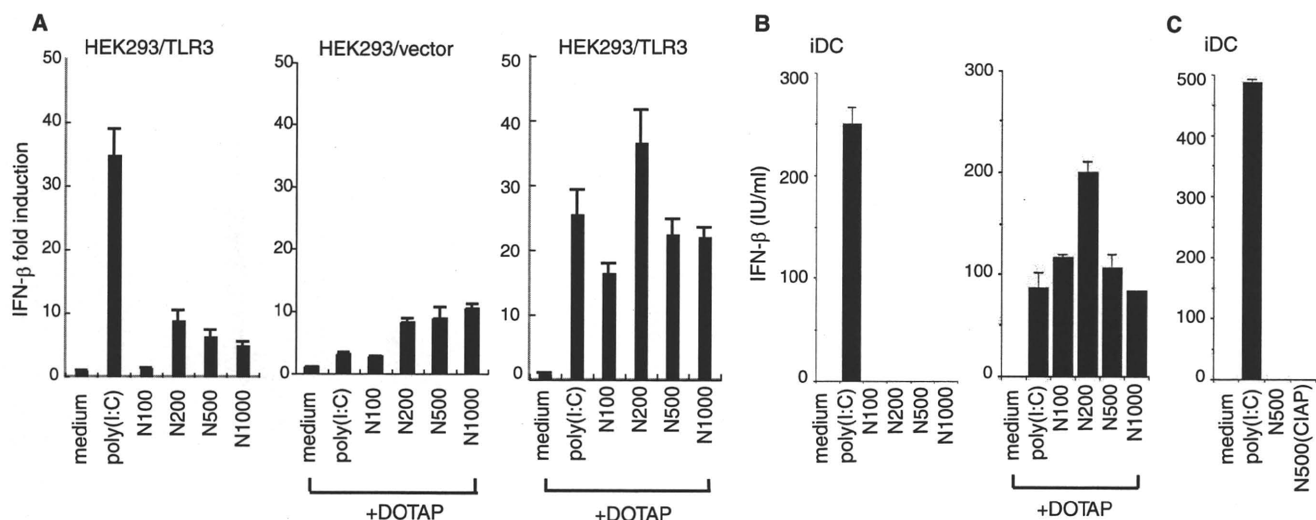


FIGURE 1. Unresponsiveness of mDCs to extracellular in vitro transcribed dsRNA. *A*, IFN-β promoter activation in HEK293 cells expressing TLR3 in response to poly(I:C) and MV-derived synthetic dsRNAs. *Left panel*, HEK293 cells in 24-well plates were transfected with pEFBOS/hTLR3 together with the reporter plasmid. Twenty-four hours after transfection, cells were stimulated with 10 μg/ml poly(I:C) or in vitro transcribed MV-originated dsRNA (N100, N200, N500, or N1000); *center and right panels*, HEK293 cells transfected with pEFBOS (*center panel*) or pEFBOS/hTLR3 (*right panel*) were stimulated with 5 μg/ml dsRNAs complexed with DOTAP. After 6 h, the luciferase reporter activities were measured and expressed as the fold induction relative to the activity of unstimulated cells. Representative data from a minimum of three separate experiments are shown. HEK293 cells transfected with pEFBOS did not respond to extracellular MV-originated dsRNAs (data not shown). *B*, IFN-β production in monocyte-derived iDCs stimulated with dsRNA. *Left panel*, Monocyte-derived iDCs (1×10^6/ml) were stimulated with 10 μg/ml poly(I:C) or in vitro transcribed MV-originated dsRNA; *right panel*, Monocyte-derived iDCs (6×10^5/ml) were stimulated with 5 μg/ml dsRNAs complexed with DOTAP and cultured for 24 h. *C*, Monocyte-derived iDCs (1×10^6/ml) were exogenously stimulated with 10 μg/ml N500 or CIAP-treated N500. After 24 h, the amount of IFN-β present in the culture supernatant was assessed by an ELISA kit. Representative data from a minimum of three separate experiments are shown.

from Amersham Bioscience. Imiquimod, Gardiquimod, CL075, ODN2006, ODN2216, ODNM362, FITC-ODN2006, and FITC-ODN2216 were purchased from Invivogen. Poly I was provided from Dr. Nishikawa (Institute for Biological Resources and Function, Tsukuba, Japan). Anti-human TLR3 mAb (clone TLR3.7) was generated in our laboratory (5). Anti-dsRNA mAb (K1) (20) was purchased from BioLink. Mouse IgG1 and mouse IgG2a were from Sigma-Aldrich, anti-CD83 mAb was from Ancell, Alexa Fluor568-conjugated goat anti-mouse IgG was from Molecular Probes, and FITC-labeled goat anti-mouse IgG was from American Qualex. Cytochalasin D was dissolved in DMSO at the concentration of 1 mg/ml.

dsRNA

dsRNAs of various lengths (N100, N200, N500, and N1000) were synthesized using a MEGAscript RNA Kit (Ambion) as described previously (21). cDNA of the N-protein of the measles virus (MV) strain Edmonston was used as a template for the transcription reaction. The synthetic dsRNAs were treated with polymyxin B (final 10 μg/ml) for 1 h at 37°C before stimulation of the cells. Treatment of dsRNA with calf intestine alkaline phosphatase (CIAP) was performed as described (22). In brief, 10 μg dsRNA was treated with 30 U CIAP (Roche) for 3 h at 37°C in the presence of 10 U RNase inhibitor (Promega). The enzyme was removed by using the RNeasy Mini kit (Qiagen).

Cytokine assay

$CD14^+$ monocytes were isolated from human PBMCs using the MACS system (Miltenyi Biotec). The monocytes were cultured for 6 days in RPMI 1640 supplemented with 10% heat-inactivated FCS and antibiotics in the presence of 500 U/ml GM-CSF and 100 U/ml IL-4 (PeproTech) to obtain monocyte-derived immature DCs (iDCs) (19). iDCs in 96-well round-bottom plates (1×10^6/ml) were stimulated with synthetic duplex RNA or poly(I:C) for 24 h in the presence of 500 U/ml GM-CSF. The amount of IFN-β present in the culture supernatants was measured by ELISA kit (TFB). In some experiments, dsRNA (1 μg) was mixed with 6 μl of *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP) (Roche) in OPTI-MEM (total 30 μl) and incubated for 10 min at room temperature. The mixture was added to iDCs (6×10^5/ml). In the case of inhibition assays, cells were preincubated with indicated concentrations of pharmacological inhibitors or nucleic acids for 1 h at 37°C and then stimulated with 10 μg/ml poly(I:C) for 24 h. At the time of supernatant collection, cells were washed twice with Dulbecco's PBS (DPBS) and then stained with propidium iodide (50 μg/ml in DPBS) for 10 min at room temperature. The viability of the cells was estimated by flow cytometry.

Assay for clathrin-dependent and independent endocytosis

iDCs and HEK293 cells were pretreated with chlorpromazine (25 μg/ml for iDCs, 50 μg/ml for HEK293 cells), methyl-β-cyclodextrin (1 mM), or medium alone for 1 h at 37°C and subsequently incubated with Alexa Fluor 488-AcLDL (0.2 μM for iDC, 0.02 μM for HEK293) or Alexa Fluor 488-CTXB (5 μg/ml for iDC, 50 μg/ml for HEK293) for 30 min at 4°C. The cells were then warmed for 5 min at 37°C to allow endocytosis to occur (23). For quenching the fluorescence of uningested Alexa Fluor 488-AcLDL or Alexa Fluor 488-CTXB, the cell suspensions were mixed with trypan blue solution (2 mg/ml in DPBS) and analyzed by flow cytometry (19).

Complementary DNA expression vector

Complementary DNA for human TLR3 was cloned in our laboratory by RT-PCR and was ligated into the cloning site of the expression vector pEFBOS, a gift from Dr. S. Nagata (Osaka University, Osaka, Japan).

Reporter gene assay

HEK293 cells (2×10^5 cells per well) seeded in 24-well plates were transiently transfected with pEFBOS/TLR3 (0.1 μg) or empty vector together with a luciferase-linked p-125 luc reporter plasmid (0.1 μg) using Lipofectamine 2000 reagent (Invitrogen). The p-125 luc reporter containing the human IFN-β promoter region (−125 to +19) was provided by Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). The total amount of transfected DNA (0.8 μg) was kept constant by adding empty vector. The plasmid phRL-TK (2.5 ng) was used as an internal control. Twenty-four hours after transfection, cells were washed and stimulated with medium alone or polymyxin B-treated dsRNA for 6 h. In some experiments, cells were incubated with dsRNA complexed with DOTAP for 6 h. In the inhibition assays, cells were preincubated with inhibitors or nucleic acids for 1 h at 37°C and then stimulated with 10 μg/ml poly(I:C) for 6 h. The cells were lysed in lysis buffer (Promega), and dual luciferase activities were measured according to the manufacturer's instructions. The firefly luciferase activity was normalized by *Renella* luciferase activitiy and is expressed as the fold induction relative to the activity of unstimulated vector-transfected cells.

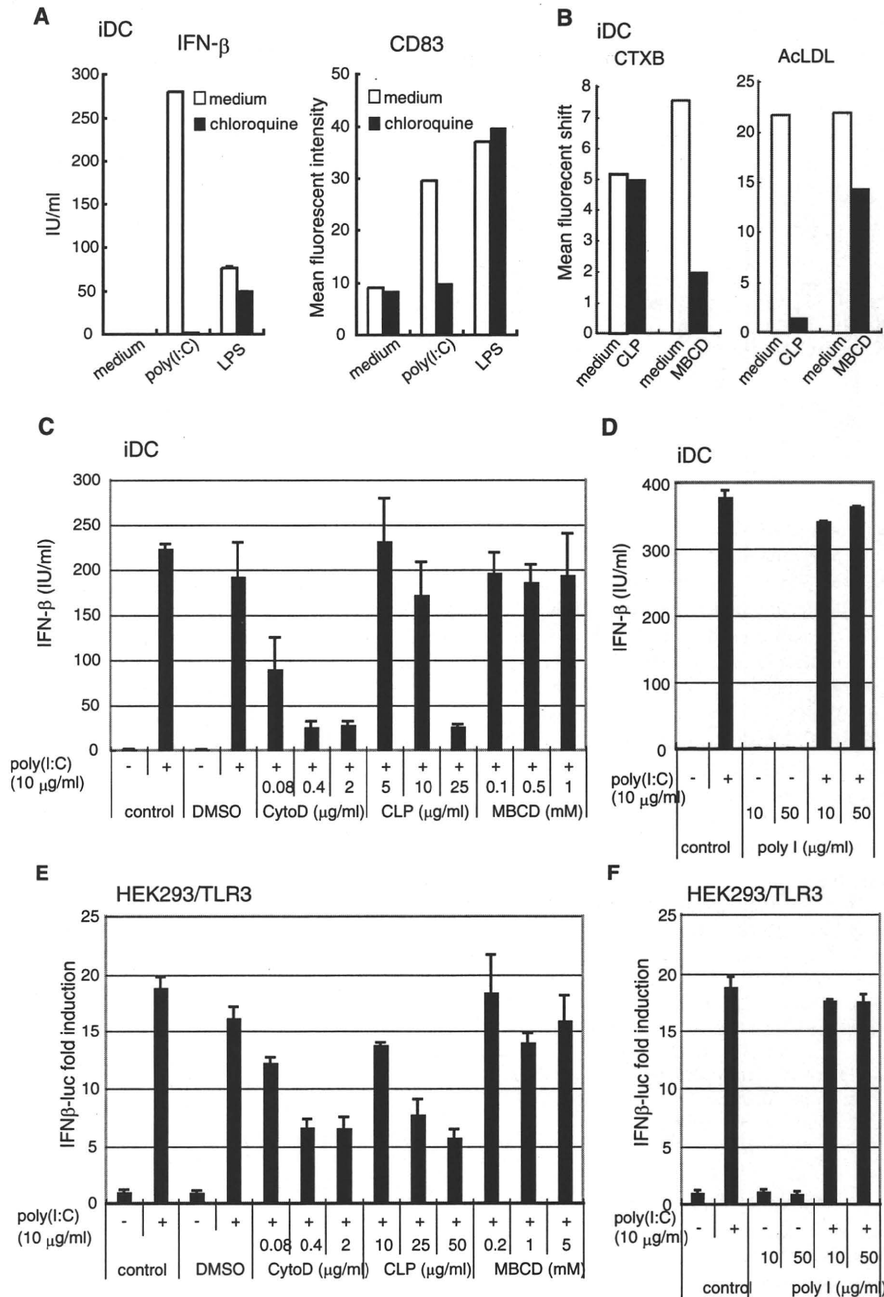


FIGURE 2. A clathrin-dependent endocytic pathway participates in poly(I:C)-induced IFN-β production. *A*, Monocyte-derived iDCs were preincubated with medium alone or 5 μg/ml chloroquine for 2 h and then stimulated with medium alone, 10 μg/ml polymyxin B-treated poly(I:C), or 100 ng/ml LPS. After 24 h, the culture medium was collected, and the amount of IFN-β was determined by an ELISA kit; the cells were then washed, and CD83 up-regulation was assessed by flow cytometry. *B*, Specificity of pharmacological inhibitors for clathrin-dependent and -independent endocytic pathways. iDCs were pretreated with 25 μg/ml chlorpromazine (CLP), 1 mM methyl-β-cyclodextrin (MBCD), or medium alone for 1 h at 37°C and subsequently incubated with 0.2 μM AlexaFluor 488-AcLDL or 5 μg/ml AlexaFluor 488-CTXB for 30 min at 4°C. The cells were then warmed for 5 min at 37°C to allow endocytosis to occur. After quenching the fluorescence of uningested AlexaFluor-488 AcLDL or AlexaFluor-488 CTXB, the cells were analyzed by flow cytometry. The similar results were obtained by using HEK293 cells (data not shown). *C*, Poly(I:C)-induced IFN-β production in monocyte-derived iDCs in the presence of inhibitors of the endocytic pathway. iDCs (1×10^6/ml) were preincubated with the indicated concentrations of cytochalasin D (CytoD), MBCD, or CLP for 1 h and then stimulated with 10 μg/ml poly(I:C). As a buffer control for CytoD, DMSO (final concentration 0.1%) was added to cells. After 24 h, the amount of IFN-β in the culture supernatant was assessed by ELISA kit. The viability of the cells was estimated with propidium iodide staining at the time of supernatant collection. iDCs: CytoD, 0 μg/ml (93.56%), 0.08 μg/ml (93.61%), 0.4 μg/ml (93.25%), 2 μg/ml (66.43%); CLP, 5 μg/ml (91.8%), 10 μg/ml (90.0%), 25 μg/ml (65.3%); MBCD, 0.1 mM (94.7%), 0.5 mM (90.0%), 1 mM (88.0%). HEK293 cells: CytoD, 0 μg/ml (93.57%), 0.08 μg/ml (90.08%), 0.4 μg/ml (81.10%), 2 μg/ml (73.24%); CLP, 10 μg/ml (93.8%), 25 μg/ml (87.0%), 50 μg/ml (86.0%); MBCD 0.2 mM (93.0%), 1 mM (86.5%), 5 mM (71.5%). *D*, iDCs were preincubated with the indicated poly I concentrations for 1 h and then stimulated with 10 μg/ml poly(I:C) for 24 h. *E* and *F*, Poly(I:C)-induced IFN-β promoter activation in TLR3-expressing HEK293 cells in the presence of inhibitors of the endocytic pathway (*E*) or poly I (*F*). HEK293 cells in 24-well plates were transfected with pEFBOS/hTLR3 together with the reporter plasmid. Twenty-four hours after transfection, the cells were washed and pretreated with the indicated concentrations of inhibitors or poly I for 1 h and then stimulated with 10 μg/ml poly(I:C). After 6 h, the luciferase reporter activities were measured and expressed as the fold induction relative to the activity of unstimulated cells. Representative data from a minimum of three separate experiments are shown.

Flow cytometry

Monocyte-derived iDCs were pretreated with medium alone or 5 μg/ml chloroquine for 2 h at 37°C and then stimulated with 100 ng/ml LPS or 10 μg/ml polymyxin B treated-poly(I:C) for 24 h. After washing, cells were incubated with mouse IgG1, or anti-CD83 mAb (1 μg) in the presence of human IgG (10 μg) for 30 min at 4°C in FACS buffer (DPBS containing 0.5% BSA and 0.1% sodium azide). After the cells were washed twice with the above buffer, FITC-labeled secondary Ab (American Qualex) was added and the cells were further incubated for 30 min at 4°C. In the case of dsRNA binding assay, cells were incubated with the indicated concentrations of poly(I:C) or N500 in culture medium for 30 min at 4°C. After washing, cells were labeled with anti-dsRNA mAb (K1) or control mouse IgG2a (1 μg) for 30 min at 4°C and then incubated with FITC-labeled secondary Ab. The cells were analyzed on a FACSCalibur (BD Biosciences). For examination of binding and internalization of ODNs, iDCs were incubated with FITC-labeled ODN2006 or ODN2216 in the presence or absence of poly(I:C) for 2 h at 37°C. After washing, cells were analyzed on FACSCalibur.

Confocal microscopy

Monocyte-derived iDCs (1×10^6/ml) were incubated with 2 μM FITC-ODN2006 for 30 min at 37°C. Cells were washed three times and treated with permeabilizing solution (BD Biosciences) for 10 min at room temperature. After washing, cells were stained with mouse IgG1 or anti-TLR3 mAb (TLR3.7) (20 μg/ml) in FACS buffer for 1 h at room temperature. Alexa Fluor 568-conjugated secondary Ab (1/400 diluted with PBS containing 10% BlocAce and 10% goat serum) was used to visualize staining of the primary Abs. Nuclei were stained with DAPI (2 μg/ml in PBS) for 10 min before mounting onto glass slides using PBS containing 2.3% DABCO and 50% glycerol. Cells were visualized at a magnification of ×63 with an LSM510 META microscope (Zeiss).

Results

Unresponsiveness of mDCs to synthetic virus-derived dsRNA

mDCs express TLR3 intracellularly and produce IFN-β in response to poly(I:C), which is a synthetic TLR3 ligand (10). To analyze the mechanism by which mDCs recognize extracellular dsRNA, we examined whether synthetic virus-derived dsRNA activates DCs to produce IFN-β. dsRNAs of various lengths were in vitro transcribed using MV cDNA encoding N-protein as the template. First, we examined the abilities of synthetic dsRNAs to activate the IFN-β promoter in HEK293 cells transiently expressing human TLR3, which predominantly express TLR3 intracellularly but possess some TLR3 molecules on the cell surface. The TLR3-acitivating abilities of these dsRNAs were remarkably lower than that of poly(I:C) when extracellularly added to the cells (Fig. 1*A*, *left panel*), while they significantly activated TLR3 when introduced into the cells using a cationic liposome DOTAP (Fig. 1*A*, *center* and *right panel*). Next, the DC-activating abilities of these dsRNAs were analyzed by measuring IFN-β production in monocyte-derived iDCs. The synthetic dsRNAs failed to induce IFN-β production in iDCs (Fig. 1*B*, *left panel*), as previously observed (21). In contrast, when dsRNA was targeted to endosomal TLR3 using DOTAP, all MV-N-derived dsRNAs induced IFN-β production at a level that was similar to or higher than that from poly(I:C) stimulation (Fig. 1*B*, *right panel*). Since these in vitro-transcribed dsRNAs contain 5′-ppp, we treated dsRNA with CIAP. Once again, extracellular CIAP-treated dsRNAs did not induce IFN-β production in iDCs, suggesting that the DC-activating ability of dsRNA is independent of its 5′ structure (Fig. 1*C*).

The clathrin-mediated endocytic pathway participates in dsRNA-induced IFN-β production

Poly(I:C)-induced IFN-β production and costimulatory molecule (CD83) up-regulation were inhibited by pretreatment of the cells with chloroquine, an inhibitor of endosomal maturation (Fig. 2*A*). The endocytic pathway that participates in TLR3-mediated signaling was analyzed using the pharmacological

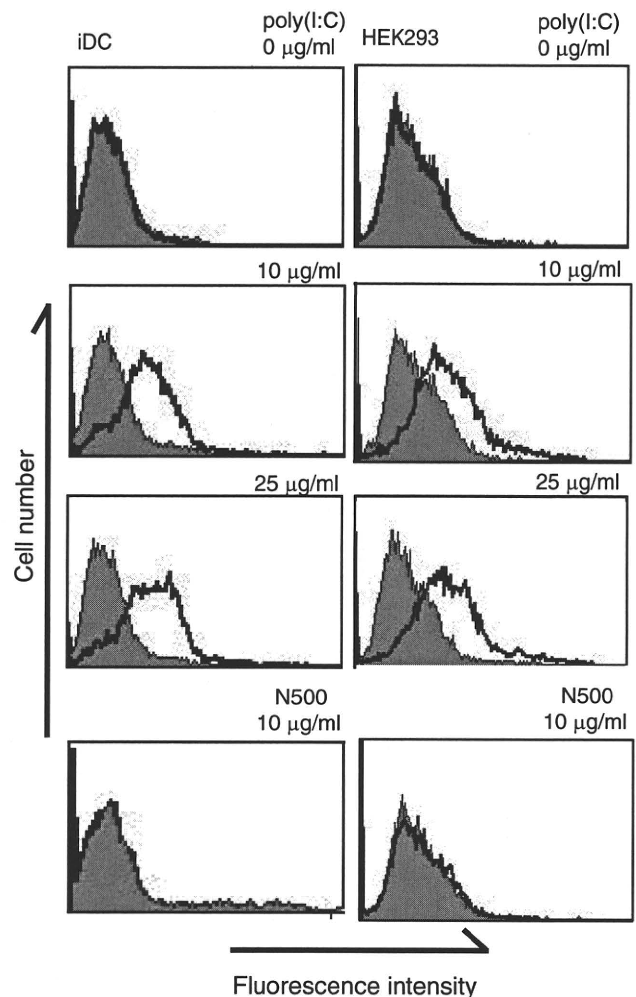


FIGURE 3. Flow cytometric analysis of poly(I:C) binding to DCs and HEK293 cells. Monocyte-derived iDCs and HEK293 cells were incubated with the indicated concentrations of poly(I:C) or N500 in the culture medium for 30 min at 4°C. After washing, the cells were stained with anti-dsRNA mAb (K1) and FITC-labeled secondary Ab (solid line). Shaded histograms represent cells labeled with isotype-matched control Ab.

inhibitors cytochalasin D (a phagocytosis inhibitor), methyl-β-cyclodextrin (a caveolae-mediated endocytosis inhibitor) (23–25), and chlorpromazine (a clathrin-mediated endocytosis inhibitor) (23, 26). We first evaluated various concentrations of these inhibitors to maximize their specificity and eliminate toxic side effects. Under the experimental setting, cell damage was negligible within the concentrations of the inhibitors used in this study. Under optimized conditions, chlorpromazine inhibited the internalization of AlexaFluor 488-AcLDL, a marker for the clathrin-dependent pathway (27), but had no effect on the uptake of AlexaFluor 488-CTXB, a marker for caveolar-mediated internalization (28). In contrast, methyl-β-cyclodextrin significantly inhibited the internalization of AlexaFluor 488-CTXB, but had few effects on AlexaFluor 488-AcLDL uptake (Fig. 2*B*). IFN-β production was inhibited by pretreatment of iDCs with cytochalasin D and chlorpromazine but not with methyl-β-cyclodextrin, suggesting that the clathrin-dependent endocytic pathway mediates cell entry of poly(I:C) to induce IFN-β gene transcription (Fig. 2*C*). In addition, poly I, an inhibitor of scavenger receptors (29), did not block poly(I:C)-induced IFN-β production in iDCs (Fig. 2*D*). Although poly I has been shown to activate TLR3 in murine B cells and bone

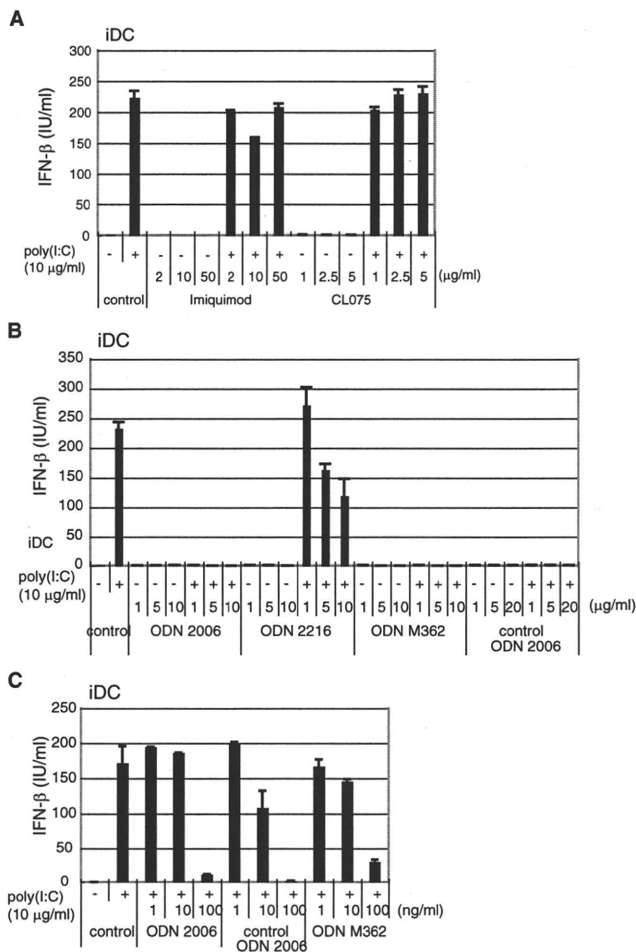


FIGURE 4. B- and C-type ODNs inhibit poly(I:C)-induced IFN-β production in DCs. Monocyte-derived iDCs (1×10^6/ml) were preincubated with the indicated concentrations of Imiquimod (TLR7 ligand), CL075 (TLR8 ligand) (*A*), ODN2006 (B-type TLR9 ligand), ODN2216 (A-type TLR9 ligand), ODNM362 (C-type TLR9 ligand), or control ODN2006 (*B* and *C*) for 1 h and then stimulated with 10 μg/ml poly(I:C). After 24 h, the amount of IFN-β in the culture supernatant was assessed by ELISA.

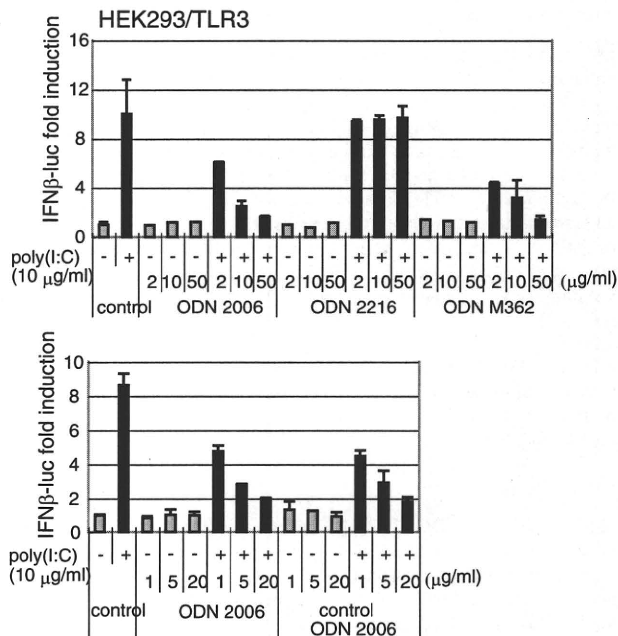


FIGURE 5. B- and C-type ODNs inhibit poly(I:C)-induced TLR3-mediated IFN-β promoter activation. HEK293 cells in 24-well plates were transfected with pEFBOS/hTLR3 together with the reporter plasmid. Twenty-four hours after transfection, the cells were washed and preincubated with the indicated concentrations of ODN2006, ODN2216, or ODNM362 for 1 h and then stimulated with 10 μg/ml poly(I:C). After 6 h, the luciferase reporter activities were measured and expressed as the fold induction relative to the activity of unstimulated cells. Representative data from a minimum of three separate experiments are shown.

marrow-derived DCs (30), human monocyte-derived iDCs could not produce IFN-β in response to extracellular poly I. Similar results were obtained with TLR3-expressing HEK293 cells (Fig. 2, *E* and *F*). Poly(I:C)-induced TLR3-mediated IFN-β promoter activation was inhibited by pretreatment of cells with cytochalasin D and chlorpromazine but not with methyl-β-cyclodextrin or poly I.

Poly(I:C) binding to mDCs

The potent IFN-β-inducing ability of poly(I:C) may be due to the uptake of the latter by iDCs. To test poly(I:C) binding to unknown cell-surface receptors, iDCs and HEK293 cells were incubated with various poly(I:C) concentrations at 4°C for 30 min. As shown in Fig. 3, poly(I:C) bound to iDCs and HEK293 cells in a dose-dependent manner. The extent of poly(I:C) binding depended on the cell type and on the individual cells in the case of iDCs. In contrast, in vitro-transcribed dsRNAs hardly bound to iDCs and HEK293 cells.

CpG ODNs but not synthetic TLR7/8 ligands inhibit poly(I:C)-induced IFN-β production in mDCs

Previous studies demonstrated that CpG ODNs are endocytosed into mice bone marrow-derived DCs (31, 32). To examine the effects of nucleic acids on poly(I:C)-induced IFN-β production, inhibition analysis was performed using synthetic ligands of TLR7, 8, and 9. iDCs were preincubated with increasing concentrations of nucleic acids for 1 h and then stimulated with poly(I:C). The TLR7/8 ligands, i.e., Imiquimod, Gardiquimod, and CL075, did not affect poly(I:C)-induced IFN-β production (Fig. 4*A*, data not shown). In contrast, the TLR9 ligands, i.e., CpG ODNs, inhibited poly(I:C)-induced IFN-β production in iDCs (Fig. 4*B*). Both B-type ODN2206 and C-type ODN M362 that induce IL-6 production and B cell proliferation completely inhibited poly(I:C)-induced IFN-β production in the 1 to 10 μg/ml range, while A-type

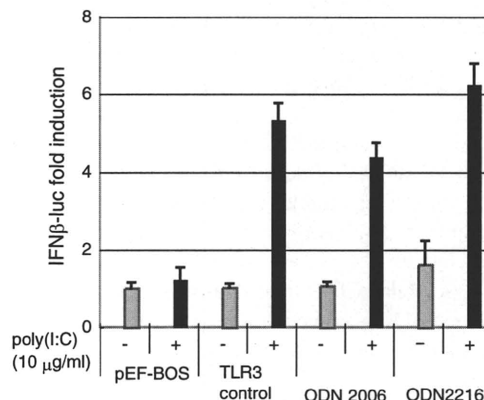


FIGURE 6. ODNs do not affect poly(I:C) recognition by endosomal TLR3. HEK293 cells in 24-well plates were transfected with pEFBOS/hTLR3 together with the reporter plasmid. Twenty-four hours after transfection, the cells were washed and stimulated with poly(I:C) (0.5 μg) and ODN2006 or ODN2216 (0.5 μg) complexed with DOTAP. After 6 h, the luciferase reporter activities were measured. Representative data from a minimum of three separate experiments are shown.

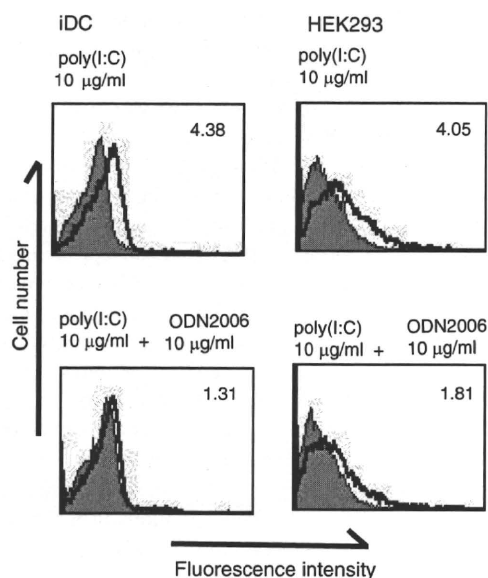
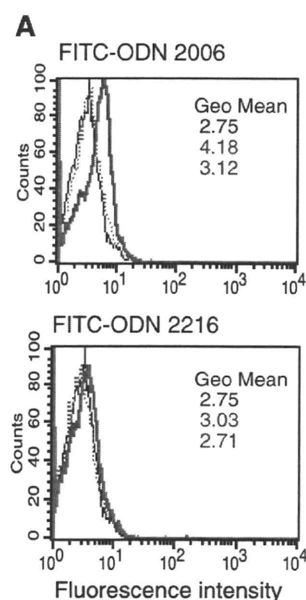


FIGURE 7. ODN2006 competes for surface binding with poly(I:C). Monocyte-derived iDCs and HEK293 cells were incubated with 10 μg/ml poly(I:C) in the presence or absence of 10 μg/ml ODN2006 for 30 min at 4°C. After washing, cells were stained with anti-dsRNA mAb (solid line) or control mouse IgG2a (shaded histograms) for 30 min at 4°C and then FITC-labeled secondary Ab. Inset values indicate the mean fluorescent intensities specific for the anti-dsRNA mAb.



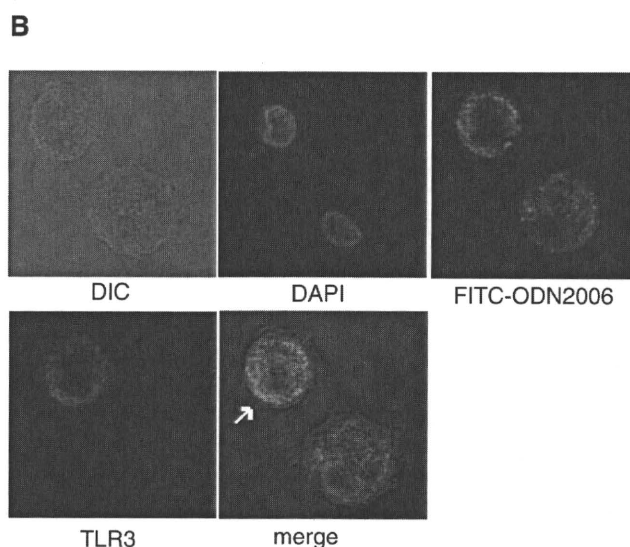


FIGURE 8. ODN2006 shares the uptake receptor with poly(I:C). *A*, Flow cytometric analysis of ODNs uptake by DCs. Monocyte-derived iDCs were incubated with FITC-ODNs (8 pmol/ml) in the presence or absence of an equal molar concentration of poly(I:C) for 2 h at 37°C. *Upper panel*, Binding and internalization of B-type ODN2006; *Lower panel*, Binding and internalization of A-type ODN2216. Black lines show self-fluorescence of the cells. Red and green lines represent the fluorescence of FITC-ODNs that are bound and endocytosed in the absence and presence of poly(I:C), respectively. Mean fluorescent intensities are shown. *B*, Confocal images of FITC-ODN2006 that has been taken up in iDCs and those of endogenous TLR3 in iDCs. iDCs were incubated with 2 μM FITC-ODN2006 for 30 min at 37°C. After washing, the cells were fixed and endogenous TLR3 was stained with anti-TLR3 mAb (TLR3.7) followed by Alexa568-labeled anti-mouse IgG. Nuclei were stained with DAPI (blue color). Green: ODN2006, Red: TLR3, Yellow: merged. Arrow indicates the colocalization of ODN2006 and TLR3. Faint TLR3 staining was observed in the lower right-hand iDC.

ODN2216 that induces robust IFN-α production in plasmacytoid DCs (pDCs) retarded poly(I:C) function in a dose-dependent manner (Fig. 4*B*). In addition, control ODN2006, a non-TLR9 ligand, also completely inhibited poly(I:C)-induced IFN-β production in a manner similar to that of ODN2006 (Fig. 4*B*), indicating that the inhibitory effect of the ODNs appears to depend on their specific sequences, not on their immunostimulatory features. In the 1 to 100 ng/ml range, ODN2006, ODN M362, and control ODN2006 inhibited poly(I:C)-induced IFN-β production in a dose-dependent manner (Fig. 4*C*). Since 10 μg/ml poly(I:C) (approximate length, 1500 bps) and 67 ng/ml ODN2006 each correspond to 8 pmol/ml, it is possible that poly(I:C) competes with ODNs for binding to a cell surface molecule that mediates endocytosis.

In contrast, the inhibitory effects of ODNs on poly(I:C)-induced TLR3 signaling in TLR3-expressing HEK293 cells were somewhat different from those in iDCs. In the 2 to 50 μg/ml range, B-type ODN2006 and C-type ODN M362 inhibited poly(I:C)-induced IFN-β promoter activation in a dose-dependent manner, while A-type ODN2216 did not affect the poly(I:C) function (Fig. 5). In TLR3-expressing HEK293 cells, it is possible that some TLR3 molecules that were present on the cell surface contribute to poly(I:C) internalization in conjunction with the uptake receptor.

Poly(I:C) recognition by endosomal TLR3 in the presence of ODNs

The inhibitory effect of ODN2006 on poly(I:C)-induced IFN-β production was abrogated when the cells were washed after preincubation with ODNs and then stimulated with poly(I:C) (data not shown). It is necessary for ODNs and poly(I:C) to be present together to inhibit poly(I:C) function. To rule out the possibility that ODNs affect TLR3-mediated poly(I:C) recognition by forming a complex with poly(I:C), poly(I:C) was introduced into the endosomal compartment together with ODNs by using DOTAP, and IFN-β production or promoter activation was measured. As shown in Fig. 6, poly(I:C) induced IFN-β promoter activation through TLR3 regardless of whether the ODNs were present or absent. These results imply that ODNs inhibit poly(I:C) function during the binding and uptake processes.

ODN2006 shares the uptake receptor with poly(I:C)

To test the possibility that B- and C-type ODN compete for surface binding with poly(I:C), iDCs and HEK293 cells were incubated with poly(I:C) in the presence or absence of ODN2006 for 30 min

at 4°C. The binding of poly(I:C) on the cell surface was assessed by flow cytometry with anti-dsRNA mAb and FITC-labeled secondary Ab. The poly(I:C) binding level was reduced in the presence of ODN2006 in both iDCs and HEK293 cells (Fig. 7). Similar results were obtained with ODNM362 (data not shown). We next analyzed the binding and internalization of FITC-labeled ODNs in iDCs by using flow cytometry. The binding and internalization of FITC-ODN2006 was observed in iDCs, and this was markedly reduced in the presence of poly(I:C) (Fig. 8*A*, *upper panel*). In contrast, FITC-labeled ODN2216 hardly bound to iDCs (Fig. 8*A*, *lower panel*), suggesting that an unknown membrane receptor mediates cell entry of ODN2006 and poly(I:C) but not that of ODN2216. Similar results were obtained with HEK293 cells (data not shown). To examine whether ODN2006 is delivered to the TLR3-positive organelle, iDCs were incubated with FITC-ODN2006 for 30 min at 37°C and then stained with anti-TLR3 mAb. Cells were analyzed by confocal microscopy. As shown in Fig. 8*B*, ODN2006 partly colocalized with TLR3.

Discussion

The mechanism by which exogenously added dsRNA can activate endosomal TLR3 is unknown. By using pharmacological inhibitors, we demonstrated that the clathrin-dependent endocytic pathway participates in dsRNA-mediated TLR3 activation. Among the synthetic dsRNAs, poly(I:C) is preferentially internalized and activates TLR3 in monocyte-derived iDCs resulting in potent IFN-β induction. In vitro transcribed MV-originated dsRNA hardly activates TLR3 when iDCs are stimulated extracellularly. Putative RNA capture at the cell surface may involve the binding and transfer of poly(I:C) but not that of MV-originated synthetic dsRNA. In addition, B- and C-type ODNs compete with poly(I:C) for cellular uptake and inhibit poly(I:C)-induced IFN-β production in iDCs in a dose-dependent manner. This indicates that in mDCs, the uptake receptor is shared by poly(I:C) and B- and C-type ODNs.

Lee et al. (16) reported that CD14 directly bound poly(I:C) small fragments and mediated the cellular uptake of poly(I:C) small fragments in mice bone marrow-derived macrophages. In the human fibroblast cell line MRC5, which expresses TLR3 and CD14 on the cell surface, poly(I:C)-induced IFN-β production was inhibited by pretreatment of the cells with chloroquine (M. Matsumoto, S. Okahira, and T. Seya, unpublished data). Anti-human TLR3 mAb (TLR3.7) inhibits poly(I:C)-induced IFN-β production in MRC5 cells, indicating that cell-surface TLR3 might be involved in poly(I:C) recognition (5). Furthermore, surface expression of both TLR3 and CD14 was decreased 30 min after poly(I:C) stimulation (M. Matsumoto, unpublished data), suggesting that CD14 and TLR3 participate in poly(I:C) uptake in human fibroblasts. In contrast, monocyte-derived iDCs and HEK293 cells do not express CD14 (19). Thus, another cell surface molecule must be involved in the cellular uptake of poly(I:C) in both cell types.

Our results showed that poly(I:C)-induced IFN-β production in iDCs was inhibited only when ODNs were also present and that the synthetic ligands for TLR7/8 did not inhibit this production (Fig. 4). Since TLR9 was not expressed on human mDCs (data not shown and Ref. 33), it is unlikely that the inhibitory effect of ODNs is caused by TLR9-mediated signaling. Additionally, all types of ODNs did not affect poly(I:C)-mediated IFN-β production when poly(I:C) and ODNs were delivered to endosomal compartments with DOTAP (Fig. 6). Finally, surface binding of poly(I:C) was reduced in the presence of B- and C-type ODNs (Fig. 7 and data not shown) and also, binding and internalization of FITC-labeled ODN2006 (type B) was decreased in the presence of an equimolar concentration of poly(I:C) (Fig. 8*A*). These results suggest that poly(I:C) shares the uptake receptor with B- and C-type ODNs in iDCs and that the inhibitory effects of B- and C-type ODNs rely on competitive binding between poly(I:C) and B- and C-type ODNs to the cell-surface receptor. In contrast, the binding and internalization of A-type ODN2216 was hardly observed in iDCs. A-type CpG ODNs (CpG-A) are potent IFN-α inducers in pDCs (34, 35). TLR9 activation by CpG-A occurs in the early endosome and leads to IFN-α production, whereas B-type CpG ODNs (CpG-B) localize to the lysosome and promote pDCs maturation (31, 32). In contrast, both CpG-A and CpG-B are delivered to the lysosome in mouse conventional DCs that express TLR9 intracellularly (31). These results imply that CpG-A and CpG-B are endocytosed through different cell surface receptors and pDCs possess specific machinery that retains the uptake receptor for CpG-A into the early endosome. In human monocyte-derived iDCs, the uptake receptor for A-type ODNs is hardly expressed, suggesting that the expression patterns of uptake receptors differ between human and mouse cells. The inhibitory effect of ODN2216 observed at excess concentrations in iDCs may reflect the weak affinity of A-type ODNs to the poly(I:C) uptake receptor.

It has been shown that poly(I:C) is delivered to the late endosome and then to the lysosome in CHO cells (16). In contrast to TLR9-MyD88 signaling, it appears that TLR3-TICAM-1 signaling does not require endosomal retention of poly(I:C). Once endosomal TLR3 is activated by poly(I:C), an adaptor molecule TICAM-1 transiently colocalizes with TLR3 and then dissociates from the receptor and forms the TICAM-1 signalosome (36). We have observed the colocalization of FITC-labeled ODN2006 and TLR3 in iDCs, indicating that the intracellular trafficking of poly(I:C) and ODN2006 is the same.

Natural and synthetic TLR7 ligands inhibit CpG-A- and CpG-C-ODN-induced IFN-α production in human pDCs (37). The TLR7 signal appears to regulate the outcome of TLR7 ligand/CpG-A-ODN costimulation. Although human mDCs express TLR8, the synthetic TLR8 ligand CL075 did not induce IFN-β production; further it did not affect poly(I:C)-induced IFN-β production in iDCs (Fig. 4*B*). Similar results were obtained with TLR3-expressing HEK293 cells and from the reporter assay for IFN-β promoter activation. The mechanisms by which extracellular TLR7/8 ligands are delivered to intracellular TLR7/8 may differ from those of TLR3/9.

Our data clearly demonstrated that mDCs possess the uptake receptor for poly(I:C) but not that for in vitro-transcribed dsRNA. B- and C-type ODNs share the internalization receptor with poly(I:C). Both poly(I:C) and ODNs are synthetic nucleic acids that do not represent natural virus products, and there are no common structural motifs. The molecular structure of nucleic acids required for recognition by the cell surface receptor should be different from that required for TLR3 activation. It is important to investigate whether virus-derived RNA activates TLR3 extracellularly. TLR3-mediated mDCs activation results in the production of IL-12p70 and IFN-α/β and DC maturation that in turn activates NK cells and CTL (38, 39). At least in mDCs in which TLR3 is localized intracellularly, the ligand properties recognized by the catch-up receptor are critical for TLR3 activation. Identification of virus-derived RNA that is recognized by both the catch-up receptor and TLR3 would clarify the in vivo function of TLR3 during viral infection.

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Disclosures

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TLR3: Interferon induction by double-stranded RNA including poly(I:C) ☆

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Abstract

Toll-like receptor 3 (TLR3) recognizes viral double-stranded RNA and its synthetic analog polyriboinosinic:polyribocytidylic acid (poly(I:C)) and induces type I interferon (IFN), inflammatory cytokine/chemokine production and dendritic cell (DC) maturation via the adaptor protein TICAM-1 (also called TRIF). TLR3 is expressed both intracellularly and on the cell surface of fibroblasts and epithelial cells, but is localized to the endosomal compartment of myeloid DCs. Several studies in TLR3-deficient mice demonstrate that TLR3 participates in the generation of protective immunity against some viral infections. Involvement of TLR3-TICAM-1 in activation of NK cells and CTLs by myeloid DCs suggests that TLR3 serves as an inducer of cellular immunity sensing viral infection rather than a simple IFN inducer. In this review, we summarize the current knowledge on TLR3 and discuss its possible role in innate and adaptive immunity.
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Keywords: Adjuvant; Cross-priming; Double-stranded RNA; Innate immunity; NK cell activation; Toll-like receptor; Type I interferon; Viral infection

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1. Introduction

Type I interferon (IFN) was first discovered in the 1950s from the independent studies by Nagano and Isaacs, in which it was demonstrated that animal cells infected with viruses

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released anti-viral factors [1,2]. Both natural and synthetic double-stranded (ds) RNAs elicit IFN production [3,4]. Several studies have suggested that human cells recognize particular spatial and steric organizations of dsRNA via putative cell membrane receptors and produce type I IFN [5–7]. Among the synthetic dsRNAs, polyriboinosinic:polyribocytidylic acid (poly(I:C)) was found to be the most potent IFN inducer [5]. In in vivo studies in mice, intraperitoneal injection of poly(I:C) elicited IFN-α/β production and NK cell activation [8,9]. However, the mechanisms by which cells recognize dsRNA and produce IFN-α/β remain largely unknown.

At the beginning of this century, Toll-like receptor 3 (TLR3), a member of the TLR family proteins, was identified as a receptor for dsRNA [10,11]. Upon recognition of dsRNA, TLR3 transmits signals via the adaptor protein Toll-IL-1 receptor (TIR) domain-containing adaptor molecule-1 (TICAM-1) (also called TIR-domain-containing adapter inducing IFN-β [TRIF]). This activates the transcription factors interferon regulatory factor 3 (IRF-3), NF-κB, and AP-1 (a complex of activating transcription factor 2 (ATF2) and JUN), leading to the induction of type I IFN (especially IFN-β), cytokine/chemokine production and dendritic cell (DC) maturation [12,13]. Although TLR3 participates in the dsRNA-induced production of type I IFN, TLR3-null cells still produce IFN-α/β in response to viral infection [14]. Additional cytosolic dsRNA receptors, retinoic-acid inducible gene-I (RIG-I) and melanoma differentiation associated antigen 5 (MDA5), have been recently identified [14,15]. RIG-I and MDA5 recognize 5′-triphosphate-containing ssRNA/dsRNA and poly(I:C), respectively and induce IFN-α/β production [16,17]. Studies using knock-out mice clearly showed that RIG-I is essential for the production of IFN-α/β in response to RNA viruses whose genomes contain 5′-triphosphate, whereas MDA5 is involved in the detection of picornaviruses [18,19]. Based on the different subcellular localization of cytosolic RNA receptors and TLR3, these receptors seem to play distinct roles in anti-viral immune responses. In this review, we summarize the current knowledge on TLR3 and discuss its possible role in innate and adaptive immunity.

2. Properties of TLR3

2.1. Structure of TLR3

Human TLR3 consists of an extracellular domain containing 23 leucine rich repeats (LRRs) and N- and C-terminal flanking regions, the transmembrane domain, and the intracellular TIR domain [20]. It possesses 15 putative carbohydrate-binding motifs in the extracellular domain. Recent structural analyses of the human TLR3 ectodomain revealed that the LRRs form a large horseshoe-shaped solenoid of which one face is largely masked by carbohydrate, while the other face is glycosylation-free [21,22]. TLR3 molecules are arranged as dimers in the crystals, and the C-terminal highly-conserved surface residues and a TLR3-specific LRR insertion at LRR20 forms a homodimer interface [21]. In addition, there are two patches of positively charged residues on the glycosylation-free surface [21]. Bell et al. demonstrated that H^{539} and N^{541} in TLR3-LRR20, located on the glycan-free lateral face, are critical amino acids for ligand binding and signaling [23]. The positively charged residues in the two patches and the carbohydrate moiety are not involved in TLR3 function [23]. Based on these results together with the structure of dsRNA, a dsRNA-induced symmetrical receptor crosslinking model has been proposed for TLR3 [23,24]. An LRR-deletion study on TLR3 suggests that the C-terminal LRRs (LRR20-LRR22) control the receptor dimerization and signaling [25]. However, it is not yet understood how extracellular receptor-receptor interactions induce structural reorganization of the cytoplasmic TIR domain, necessary for TICAM-1 binding and activation.

2.2. Expression and subcellular localization of TLR3

Human *TLR3* mRNA has been detected in the placenta, pancreas, lung, liver, heart and brain [26]. It is also expressed in myeloid DCs and intestinal epithelial cells but not in monocytes, polymorphonuclear leukocytes, T, B and NK cells, suggesting a physiological role in innate immunity [27–29]. In addition, the second type of DC precursor cell, pre-DC2 (previously known as plasmacytoid DC precursor), which expresses TLR7 and TLR9 and secretes large amounts of IFN-α in response to ssRNA and imidazoquinoline compounds (TLR7 ligands) or CpG DNA (TLR9 ligand), does not express TLR3 [30,31]. These observations suggest that these DC subsets have different roles in anti-viral immune responses.

It has been shown using flow cytometry with anti-human TLR3 mAb (TLR3.7) that human fibroblasts and epithelial cells express TLR3 both intracellularly and on the cell surface while monocyte-derived immature DCs and $CD11c^{+}$ blood DCs only express TLR3 intracellularly [11,31]. TLR3.7 mAb inhibits poly (I:C)-induced IFN-β production by fibroblasts, indicating that the cell-surface TLR3 participates in the poly(I:C) recognition [31]. However, in both fibroblasts and myeloid DCs, TLR3 signaling arises in the intracellular compartment, requiring endosomal maturation [31]. Immunofluorescent staining and confocal microscopic analysis of myeloid DCs and human embryonic kidney (HEK) 293 cells stably expressing human TLR3 revealed that TLR3 localizes to specific unidentified intracellular vesicles [32]. More recently, it has been shown that some TLR3 molecules localize to the early endosome in epithelial cell lines [33,34]. The structural motifs determining the intracellular localization of TLR3 are located in the 'linker' region between the transmembrane domain and the TIR domain of TLR3 [32,35]. This differs from the regulatory mechanism of intracellular TLR7 and TLR9 [35,36]. Interestingly, murine embryonic fibroblasts (MEFs) do not respond to exogenous addition of poly(I:C), suggesting that cell-surface expression of TLR3 is species-specific in fibroblasts.

In macrophages, DCs and epithelial cells, TLR3 expression is up-regulated by viral infection and exogenous addition of poly (I:C) or type I IFN [37–39]. In addition, TLR3 expression in human astrocytes is increased by poly(I:C) stimulation [40,41]. The IFN-responsive element (ISRE) located at approximately −30 bp on the human *TLR3* promoter region is responsible for viral- and poly(I:C)-induced *TLR3* gene expression [38,39]. Released IFN-α/β acts on DCs in an autocrine manner through

IFN-α/β receptor to induce TLR3 expression. Even in this case, TLR3 expression is up-regulated intracellularly but not on the cell surface in DCs. Importantly, the regulation of murine TLR3 expression is somewhat different from that of human TLR3 [38]. Although lipopolysaccharide strongly induces TLR3 expression in mouse macrophages and DCs [10,38], this effect is not observed in human cells. The sequences of the proximal promoter regions as well as the non-coding 5′-exons are different in these two species [42]. Despite the overall characteristic difference in *TLR3* promoter sequences, mRNA expression of *TLR3* is induced by type I IFN in both mice and humans.

2.3. TLR3 ligands

TLR3 recognizes both in vitro-transcribed dsRNA and poly (I:C), suggesting that the RNA duplex and not 5′-triphosphate is critical for TLR3 activation [10,11,43]. Since TLR3 is predominantly expressed intracellularly, ligands are often transfected into the cells with cationic liposomes such as lipofectin or DOTAP. dsRNA–liposome complexes are thought to be delivered to the endosome where they activate TLR3. Under such experimental conditions, bacterial total RNA and in vitro-transcribed ssRNA but not mammalian total RNA, which is abundant in modified nucleosides, activate HEK293 cells expressing TLR3 [44]. By using modified RNAs, it has been shown that the recognition of in vitro-transcribed ssRNA by TLR3 is suppressed by modification of nucleotides such as methylation probably due to destabilization of RNA duplexes [44]. Although TLR3 appears to recognize the dsRNA structure formed in unmodified RNA, the possibility that the RIG-I/MDA5 pathway participates in the recognition of these RNA cannot be excluded. It would be interesting to determine whether viral RNA derived from negative-stranded or positive-stranded ssRNA viruses are recognized by TLR3. Precise studies using null cells are necessary for identification of natural ligands for TLR3.

2.4. Delivery of dsRNA

TLR3 is activated by extracellular dsRNA. The mechanisms by which extracellular dsRNA is delivered to the TLR3-containing organelle remain unknown. A recent study demonstrated that CD14 enhances dsRNA-mediated TLR3 activation by directly binding to poly(I:C) and mediating cellular uptake of poly (I:C) [45]. The internalized poly(I:C) colocalizes with CD14 and TLR3. Since the extracellular domain of CD14 consists of LRRs [46], poly(I:C) might be transferred from CD14 to TLR3. In human fibroblasts, cell-surface TLR3 is involved in the recognition of dsRNA [11]. Since CD14 is expressed on the fibroblast cell surface, it may cooperate with TLR3 to internalize dsRNA. On the other hand, in the case of CD14-negative myeloid DCs, extracellular dsRNA must be internalized with the putative uptake receptor. Remarkably, uptake of dsRNA largely depends on the dsRNA structure. Among various synthetic dsRNAs, poly(I:C) is preferentially internalized and activates TLR3 in myeloid DCs [43]. High dose of poly(I:C) reportedly activates MDA5 and induces type I IFN production [18,19]. However, it is not known how poly(I:C) reaches the cytosol. Physiologically, TLR3 may encounter viral RNAs in the endosome where viruses enter through the endocytic pathway or by uptake of the apoptotic bodies derived from virally infected cells. Cells infected by positive-stranded RNA viruses and DNA viruses contain virus-derived dsRNA [47]. Indeed, Ebihara et al., recently reported that myeloid DCs phagocytosed the apoptotic bodies derived from Hepatitis C virus-infected cells containing HCV-derived dsRNA and induced innate immune responses including IFN-β production [48].

3. The TLR3-mediated type I IFN signaling pathway

Among the TLR family members, only TLR3 does not use myeloid differentiation factor 88 (MyD88) as a signaling adaptor [49]. TLR3 mediates signals via the adaptor protein TICAM-1/TRIF [12,13] (Fig. 1). 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. The TIR domain of TLR3 is responsible for signaling and recruiting the adaptor protein. A substituted mutant A795H, in which an alanine residue in the B–B loop of the TIR domain of TLR3 is replaced with a histidine residue [50], does not activate NF-κB and the IFN-β promoter in HEK293 cells upon poly(I:C) stimulation because of its failure to bind TICAM-1 [12]. In addition, phosphorylation of two specific tyrosine residues (Tyr^{759} and Tyr^{858}) in the TIR domain of TLR3 is essential for

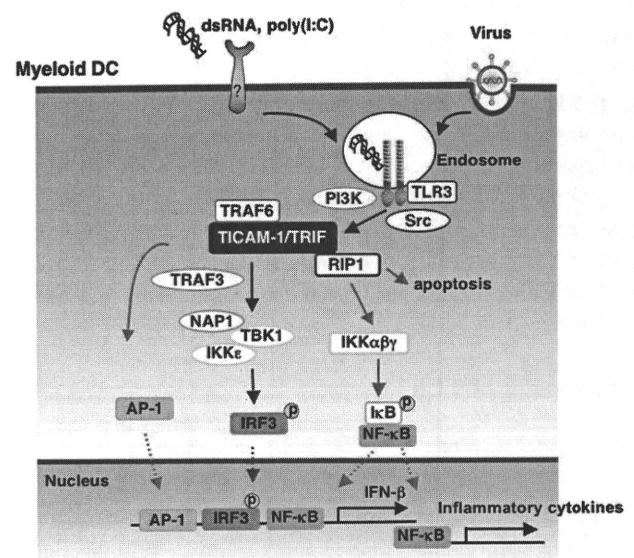

Fig. 1. TLR3-TICAM-1-mediated type I IFN signaling pathway. In myeloid DCs, TLR3 is expressed in the endosomal compartments and recognizes extracellular viral dsRNA and its synthetic analog poly(I:C). Once TLR3 is dimerized by internalized dsRNA, it recruits the adaptor protein TICAM-1/TRIF. After the transient association of TLR3 with TICAM-1 through the TIR domains, TICAM-1 dissociates from TLR3 to form a speckle-like structure containing RIP1, TRAF3 and NAP1 where TICAM-1-mediated signaling is initiated. 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 signaling. 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.

dsRNA-induced signaling [51]. Phosphatidylinositol 3-kinase (PI3-K) is recruited to these residues, and is required for full phosphorylation and activation of IRF-3. TLR3 also associates with c-Src tyrosine kinase on the endosome in response to dsRNA [52]. dsRNA-induced IRF-3 activation is abolished in Src kinase deficient cells, suggesting a critical role for Src kinase in dsRNA-TLR3-mediated signaling [52]. The Src kinase inhibitor markedly inhibits dsRNA-elicited phosphorylation of Akt, a downstream target of PI3-K. Although these data suggest that IRF-3 activation via the PI3-K-Akt-pathway is dependent on c-Src, the precise role of c-Src requires further elucidation.

TICAM-1 consists of an N-terminal proline-rich domain, a TIR domain and a C-terminal proline-rich domain. 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 [TRAM]) [53,54]. Once TICAM-1 is oligomerized, the serine-threonine kinases, TANK-binding kinase 1 (TBK-1; also called NAK or T2K) and IκB kinase-related kinase ε (IKK-ε; also called IKK-i), are activated and phosphorylate IRF-3 [55,56]. Phosphoprylated IRF-3 translocates into the nucleus and induces *IFN-β* gene transcription [57]. The N-terminal deletion mutant of TICAM-1 abolishes the *IFN-β* promoter activation while sustaining the NF-κB activating ability, suggesting an important role for the N-terminal region in TICAM-1-mediated IRF-3 activation [12]. It is reported that 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 [58]. In addition, TRAF3 is involved in the TLR3-TICAM-1-mediated IRF-3 activation [59,60]. Cells lacking TRAF3 are defective in IFN-β production but not NF-κB activation in response to poly(I:C). Although both NAP1 and TRAF3 associate with 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.

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 (RHIM) domain in the C-terminal region and acts as an NF-κB inducer and apoptosis mediator in TICAM-1-mediated signaling [61–63]. TRAF6 has also been implicated in NF-κB activation by TICAM-1 [64]. TRAF6 directly binds to the N-terminal region of TICAM-1 through the TRAF domain. Although TRAF6 is required for NF-κB activation in MEFs [65], poly(I:C)-induced NF-κB activation is not impaired in TRAF6-deficient macrophages [66]. The participation of TRAF6 in TICAM-1-mediated signaling may depend on the cell types.

Recently, Funami et al. reported subcellular localization of TICAM-1 and its dynamics in response to dsRNA [33]. TICAM-1 alters its distribution profile from diffuse to a speckle-like structure in response to poly(I:C) stimulation. TICAM-1 is transiently recruited to the endosomal TLR3 in response to poly(I:C), and thereafter moves away from TLR3 to form speckle-like structures. The downstream signaling molecules RIP1 and NAP1 are also recruited to the TICAM-1-positive speckles. Hence, TICAM-1-mediated signaling events are closely associated with the spatiotemporal mobilization and speckle formation of TICAM-1.

TLR3-TICAM-1-mediated signaling is negatively regulated by a fifth TIR adaptor protein SARM [67]. 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.

4. Anti-viral function

Viral infections result in the stimulation of IFN-α/β and IFN-inducible genes, which play a critical role in anti-viral host defense [68]. Since TLR3 responds to a synthetic dsRNA, poly (I:C), and viral dsRNA and induces IFN-β gene transcription, it is thought that TLR3 plays a key role in anti-viral immune responses. However, a study in TLR3-deficient ($TLR3^{-/-}$) mice showed that the immune response to different viruses including lymphocytic choriomeningitis virus (LCMV, a positive sense single-stranded (ss) RNA virus), vesicular stomatitis virus (VSV, a negative sense ssRNA virus), murine cytomegarovirus (MCMV, a dsDNA virus) and reovirus (a dsRNA virus), was unaffected compared to wild-type mice [69] (Table 1). In contrast, Wang et al. demonstrated the important role of TLR3 in infection by West Nile virus (WNV, a positive sense ssRNA virus) [70]. $TLR3^{-/-}$ mice had impaired cytokine production and enhanced viral load in the periphery, whereas in the brain, viral load, inflammatory responses and neuropathology were reduced compared to wild-type mice [70] (Table 1). 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.

Table 1
Representative results from TLR3-deficient mice studies on viral infection

Virus	Genome structure	Phenotype in $TLR3^{-/-}$ mice	Reference
MCMV	dsDNA	Survival ↘ Vial load in spleens ↑ IFN-α/β, IL-12 and IFN-γ in serum ↓ NK and NKT cell activation ↓	[76]
LCMV	ssRNA (−)	Normal $CD4^+$ or $CD8^+$ T cell responses (IFN-γ production) Normal T cell memory function	[69]
VSV	ssRNA (−)	Normal $CD4^+$, $CD8^+$ T cell responses (IFN-γ production)	
MCMV	dsDNA	Normal $CD4^+$, $CD8^+$ T cell responses (IFN-γ production)	
Reovirus	dsRNA	CNS injury, mortality (no difference)	
WNV	ssRNA (+)	Survival ↑ Viral load in blood ↑ IL-6, TNF-α, IFN-β ↓ Viral load in brain ↓ Neuropathology ↓	[70]
RSV	ssRNA (−)	Pulmonary mucus production ↑ Pulmonary IL-13 expression ↑	[73]
IAV	ssRNA (−)	Survival ↑ Viral load in lungs ↑ IL-6, IL-12 and RANTES ↓ $CD8^+$ T cells in lung ↓	[74]

Therefore, TLR3$^{-/-}$ mice are more resistant to lethal WNV infection. In this case, TLR3 responds to viral infection but does not display a protective role.

In other RNA viral infections such as respiratory syncytial virus (RSV), influenza A virus (IAV), and phlebovirus, TLR3-dependent inflammatory cytokine and chemokine production also appears to affect the virally induced pathology and host survival [71–75]. TLR3$^{-/-}$ mice infected by IAV had reduced inflammatory mediators leading to increased survival [74]. It is notable that these experimental conditions that use higher viral doses may lead to over-production of inflammatory cytokines and chemokines.

Interestingly, a TLR3-mediated anti-viral response has been demonstrated in MCMV infection [76]. 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-deficient mice. Thus, the role of TLR3 in the anti-viral response appears to be dependent on the viral genome structure, entry route into the cells, viral affecting sites, and property of the host anti-viral effector functions.

5. Induction of adaptive immunity

5.1. Induction of cross-priming by TLR3

Selective TLR3 expression in myeloid DCs but not in plasmacytoid DCs raises the possibility that TLR3 may play a key role in the anti-viral response by induction of the adaptive immune responses rather than primary IFN-α/β production. Myeloid DCs are the best professional antigen presenting cells having several antigen processing and transporting pathways [77]. One of the most notable features of myeloid DCs is a cross-presentation of exogenous antigens to CD8^{+} T cells (Fig. 2). This pathway is important for effective host CTL induction against viruses that do not directly infect DCs. DCs take up cell-associated antigens and after processing, present peptides bound to MHC class I molecules to CD8^{+} T cells. Several mechanisms have been proposed to explain cross-presentation [78]. Virus-induced type I IFN also promotes cross-priming of CD8^{+} T cells through direct stimulation of DCs, although the downstream signal involved in this pathway is unknown [79]. Datta et al. demonstrated that TLR3 and TLR9 ligands induced cross-presentation by bone marrow-derived DCs in a transporter associated with antigen processing (TAP)-dependent manner [80]. Using TLR3-deficient mice, Schiltz et al. clearly showed that TLR3 has an important role in cross-priming [81]. Murine CD8α^{+} DCs, which express TLR3 and have a central role in cross-presentation, 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 (OVA) antigen, induces OVA-specific CD8^{+} T cell responses, which are largely dependent on TLR3-expressing DCs [81]. In this study virus that could not infect APCs and release vial progeny from infected cells was used to avoid direct CTL priming and the effects of virus-induced IFN-α/β in vivo. In many cases, virally infected cells produce IFN-α/β which activate DCs to promote CD8^{+} T cell cross-priming [79]. Thus, both TLR3- and IFN-α/β-mediated signaling are likely implicated in licencing DCs for cross-priming of CD8^{+} T cells.

5.2. TLR3-TICAM-1-mediated NK activation

CTL and NK cells are principal effector cells in anti-viral and anti-tumor immunity. Contribution of TLR3 and TLR9 in anti-viral responses has been shown in MCMV infection [76,82], which partly relies on NK cell activation for virus clearance. Production of type I and type II IFNs as well as NK cell activation after MCMV infection is impaired in TLR3$^{-/-}$ mice and TLR9 mutant mice, suggesting that TLR3- and TLR9-mediated type I IFNs activate NK cells. Recently, a critical role of CD11c^{high} DCs in NK cell activation has been demonstrated in vivo [83]. Recruitment of NK cells to local lymph node after TLR3 stimulation and IL-15 released by DCs in a IFN-α/β-dependent manner are necessary and sufficient for the priming of NK cells, resulting in IFN-γ production and cytotoxicity against target cells [83]. Akazawa et al. subsequently showed that the TLR3 adaptor TICAM-1 is essential for poly(I:C)-induced NK cell-mediated tumor regression in a syngeneic mouse tumor implant model [84]. Remarkably, production of

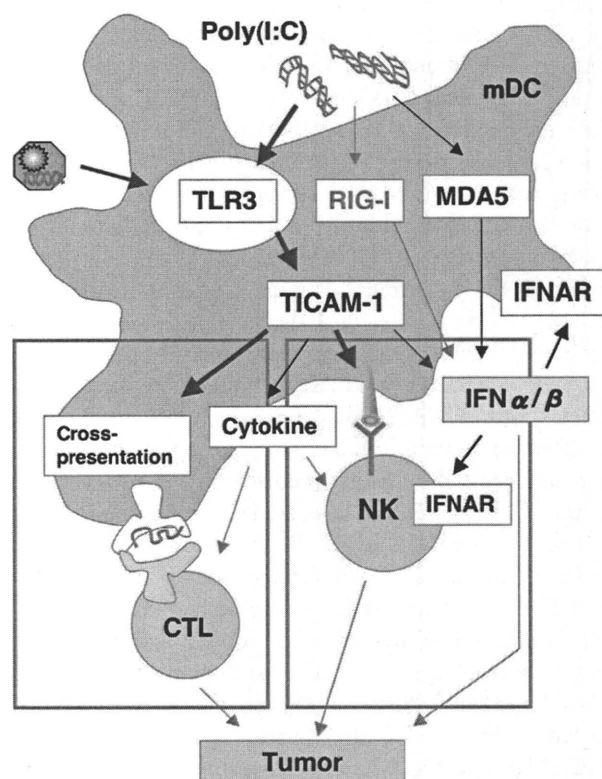

Fig. 2. dsRNA-induced TLR3-TICAM-1-mediated cellular responses in myeloid DCs. Myeloid DCs take up extracellular poly(I:C) or apoptotic bodies from virally infected cells and induce type I IFN and cytokine production, NK cell activation and CTL induction via the TLR3-TICAM-1 pathway. Extracellular poly(I:C) also activates the cytosolic RNA helicase MDA5 and induces robust type I IFN production. Type I IFN participates in NK cell activation and promotion of cross-priming resulting in the CTL induction.

IFN-α is not impaired in TICAM-1$^{-/-}$ mice compared to wild-type mice, after in vivo poly(I:C) injection or in vitro mDC stimulation, whereas IL-12 production is completely dependent on TICAM-1, consistent with previous reports [18]. Furthermore, NK cell activation requires cell–cell contact with mDCs preactivated by poly(I:C) but not IFN-α or IL-12. Thus, the TLR3-TICAM-1 pathway in mDCs facilitates the mDC–NK cell interaction following NK cell activation (Fig. 2). Poly(I:C)-induced TICAM-1-independent IFN-α production, which does not contribute to NK cell activation in this case, probably relies on the cytosolic dsRNA receptor MDA5. It is not presently known whether the RIG-I/MDA5-signaling is implicated in mDC-mediated NK cell priming. Further, identification of putative NK cell activating molecules inducible on mDCs by TICAM-1-signaling remains undetermined.

5.3. Application of the TLR3 ligand to adjuvant vaccine therapy

Until now, application of the TLR3 ligand to adjuvant vaccine therapy has been tried in a syngeneic mouse tumor implant model or in a viral infection mouse model [84–87]. Selective expression of TLR3 in mDCs and TLR3-TICAM-1-induced immune responses (type I IFN, cytokine/chemokine production, DC maturation, CTL and NK cell activation) are advantages associated with using TLR3 ligands as an adjuvant. However, several issues remain unresolved including a suitable transport system for TLR3 ligands. Poly(I:C) intraperitoneally injected in mice activates both TLR3 and MDA5 [18,84], indicating that extracellular poly(I:C) is delivered to endosomal TLR3 and further to cytosolic MDA5 in murine cells. The mechanism of intracellular transport of poly(I:C) has not been analyzed. In human monocyte-derived immature DCs, IFN-β production in response to extracellular dsRNA largely depends on the dsRNA structure [43]. Extracellular addition of DCs fails to produce IFN-β in response to in vitro-transcribed dsRNA, suggesting that the internalization of dsRNA in mDCs depends on the dsRNA structure. Thus, the dsRNA structure and targeting approach of dsRNA to the endosomal TLR3 in mDCs are important matters for generating the innate and adaptive immune responses by TLR3 ligands. Goen et al. 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 [87]. Identification of the putative dsRNA uptake receptor is crucial for analyzing the intracellular transport of dsRNA. Furthermore, clarification of the difference between the RIG-I/MDA5-mediated and TLR3-TICAM-1-mediated signaling pathways is important for assessment of the dsRNA-induced immune responses.

6. Conclusion

Numerous studies on TLR3 have been performed in the past 10 years. Although it is clear that TLR3 recognizes extracellular dsRNA and induces TICAM-1-mediated innate and adaptive immunity, the in vivo role in anti-viral responses is still controversial. In addition, the mechanisms by which endosomal TLR3 and intracellular MDA5 recognize extracellular dsRNA are not fully understood. Identification of the TLR3-TICAM-1-mediated signaling cascades different from those of RIG-I/MDA5, ligand repertoire of TLR3, and dsRNA transport system provides new insights for understanding the TLR3 function in vivo, as well as for development of the TLR3-related vaccine adjuvant for tumor and/or infectious diseases.

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