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Subcellular Localization of Toll-Like Receptor 3 in Human Dendritic Cells¹

Misako Matsumoto,^{2,*†¶} Kenji Funami,^{*†¶} Masako Tanabe,^{*†¶} Hiroyuki Oshiumi,^{*†¶} Masashi Shingai,[‡] Yoshiyuki Seto,[‡] Akitsugu Yamamoto,[§] and Tsukasa Seya^{*†¶}

Toll-like receptor (TLR)3 recognizes dsRNA and transduces signals to activate NF- κ B and IFN- β promoter. Type I IFNs (IFN- α/β) function as key cytokines in anti-viral host defense. Human fibroblasts express TLR3 on the cell surface, and anti-TLR3 mAb inhibits dsRNA-induced IFN- β secretion by fibroblasts, suggesting that TLR3 acts on the cell surface to sense viral infection. In this study, we examined the expression and localization of human TLR3 in various DC subsets using anti-TLR3 mAb. In monocyte-derived immature dendritic cells (iDCs), TLR3 predominantly resided inside the cells but not on the cell surface. iDCs produced IL-12p70 and IFN- α and - β in response to poly(I:C). Similar response was observed in iDCs treated with rotavirus-derived dsRNA. These responses could not be blocked by pretreatment of the cells with anti-TLR3 mAb. In CD11c⁺ blood DCs, cytoplasmic retention of TLR3 was also observed as in monocyte-derived iDCs, again endorsing a different TLR3 distribution profile from fibroblasts. In precursor DC2, however, TLR3 could not be detected inside or outside the cells. Of note, there was a putative centrosomal protein that shared an epitope with TLR3 in myeloid DCs and precursor DC2, but not peripheral blood monocytes. Immunoelectron microscopic analysis revealed that TLR3, when stably expressed in the murine B cell line Ba/F3, was specifically accumulated in multivesicular bodies, a subcellular compartment situated in endocytic trafficking pathways. Thus, regulation and localization of TLR3 are different in each cell type, which may reflect participation of cell type-specific multiple pathways in antiviral IFN induction via TLR3. *The Journal of Immunology*, 2003, 171: 3154–3162.

Toll-like receptors (TLRs)³ recognize pathogen-associated molecular patterns, and induce antimicrobial immune responses (1–4). Ten members of the TLR family protein (TLR1–10) have been identified in humans (5, 6). Each TLR recognizes a distinct microbial component and elicits different, but sometimes overlapping, immune responses (4). Several studies showed that immunocompetent cells differentially express TLR members and their levels were regulated in a cell type-specific manner (7–9). TLR3 recognizes dsRNA of viral origin (10, 11) and is most similar to TLR9, which recognizes bacterial CpG DNA (12). Human TLR3 is selectively expressed in dendritic cells (DCs), fibroblasts, and intestinal epithelial cells, playing physiological roles in antiviral innate immunity (7–9, 11). TLR3 also recognizes polyriboinosinic:polyribocytidylic acid poly(I:C), a

synthetic analog of dsRNA, and transduces signals to activate NF- κ B and IFN- β promoter via myeloid differentiation factor 88 (MyD88)-independent and MyD88 adapter-like/Toll-IL-1R domain (TIR) adapter protein-independent manner (11, 13, 14). A novel adapter molecule named TIR-containing adapter molecule (TICAM)-1 was identified, which binds the TIR of TLR3 and transmits signal to activate IFN- β promoter in response to poly(I:C) (14). Human lung fibroblast cell line, MRC-5, expresses TLR3 on the cell surface, and anti-TLR3 mAb we established inhibited poly(I:C)-induced IFN- β production in MRC-5 cells, which suggest that TLR3 may act on the cell surface to sense viral infection (11).

Type I IFNs (IFN- α/β) play a critical role in antiviral immune responses, and also mediate a variety of immunoregulatory effects that may link between innate and adaptive immune responses (15, 16). IFN- β is mainly produced by fibroblasts upon viral infection or treatment with poly(I:C), while human plasmacytoid precursor DC (pre-DC)2, known as natural IFN-producing cells, is a major source of IFN- α upon exposure to viruses and bacteria (17, 18). Monocyte-derived immature DCs (iDCs), on the other hand, produce large amounts of IL-12p70 and up-regulate costimulatory molecules on their cell surface by treatment with dsRNA, which induces Th1 differentiation leading to antiviral immune responses (19, 20). Recent studies demonstrated that TLR3 was expressed in monocyte-derived iDCs and CD11c⁺ immature blood DCs, but not in pre-DC2 at the message level (7, 9, 21, 22). Although myeloid DCs have TLR3 and sense dsRNA, how DCs recognizes dsRNA by their TLR3 remains unknown.

In this study, using a mAb against human TLR3, we analyzed the expression and localization of TLR3 in human DC subsets. We show evidence suggesting that regulation and localization of TLR3 expression are different in each cell type and that intracellular TLR3 responds to dsRNA in iDCs.

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³ Abbreviations used in this paper: TLR, Toll-like receptor; DC, dendritic cell; iDC, immature DC; pre-DC; precursor DC; MALP-2, macrophage-activating lipopeptide-2; MVBs, multivesicular bodies; BDCA, blood DC Ag; ODN, oligodeoxynucleotide; PKR, protein kinase R.

Materials and Methods

Cells and reagents

CD14⁺ monocytes were isolated from human PBMCs using the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). The monocytes were cultured for 6 days in RPMI 1640 supplemented with 10% heat-inactivated FCS (JRH Biosciences, Lenexa, KS) and antibiotics, in the presence of 500 U/ml GM-CSF (PeproTech EC, London, U.K.) and 100 U/ml of IL-4 (PeproTech) to obtain monocyte-derived iDCs (23). To induce the maturation of iDCs, the cells were cultured for 40 h with 10 ng/ml LPS from *Escherichia coli* serotype 0111:B4 (Sigma-Aldrich, St. Louis, MO), 100 nM of synthetic macrophage-activating lipopeptide 2 (MALP-2) (24, 25), or 12.5 μg/ml poly(I:C) (Amersham Pharmacia Biotech, Piscataway, NJ) in the presence of 500 U/ml GM-CSF. MALP-2 and poly(I:C) were treated with polymyxin B (Sigma-Aldrich) (final concentration, 10 μg/ml) for 1 h at 37°C before stimulation of the cells. Human CD11c⁺ blood DCs and plasmacytoid pre-DC2 were isolated from PBMCs using a blood DC Ag (BDCA)-1 cell isolation kit or BDCA-4 cell isolation kit, respectively, according to the manufacturer's instructions (Miltenyi Biotec). The purity of BDCA-1⁺ myeloid blood DCs and BDCA-4⁺ plasmacytoid blood DCs was >95%. HeLa cells were cultured in MEM containing 5% heat-inactivated FCS and antibiotics. The IL-3-dependent murine Ba/F3 cells were cultured in RPMI containing 10% heat-inactivated FCS, 5 ng/ml murine IL-3, 100 μM 2-ME, and antibiotics. Recombinant human IFN-β was kindly provided by Toray (Tokyo, Japan). The phosphorothioate CpG oligodeoxynucleotide (ODN) 2006 (TCGTCGTTTGTGCTTTTGTCGT) was synthesized by Hokkaido System Science (Hokkaido, Japan).

Stable transfectant

Stable transfectants expressing human TLR3 were prepared as previously described (11). Briefly, Ba/F3 cells were transfected with the pEFBOS expression vector encoding human TLR3, together with pSV2neo plasmid by electroporation. The transfectants were selected with G418 for 10 days. The expression of TLR3 was confirmed by intracellular staining for the flag epitope, which had been attached to the COOH terminus of TLR3.

RT-PCR analysis

Total RNA was isolated from monocytes, monocyte-derived iDCs, BDCA-1⁺ myeloid blood DCs, and BDCA-4⁺ plasmacytoid DCs using RNeasy Mini kit (Qiagen, Valencia, CA). DNase I-treated total RNA (3 μg) was reverse-transcribed by using random primers with RNaseH-free reverse transcriptase (Invitrogen, San Diego, CA). TLR2, TLR3, TLR4, and GAPDH were amplified using specific primers described below. TLR2 (5'-GGCTCTGGTGTGACATCC-3'/5'-CCAACTCCATTAAGGGTACAGTC-3'); TLR3 (5'-CTCAGAAGATTACCAGCCGCC-3'/5'-CCATTA TGACAGATCTAATG-3'); TLR4 (5'-ACCCATCCAGAGTTTAG CCC-3'/5'-GTACACTCACCAGGAAAATG-3'); GAPDH (5'-CACA GTCCATGCCATCACTG-3'/5'-TACTCCTTGGAGGCCATGTG-3').

Monoclonal Abs

Anti-human TLR2 mAb (TLR2.45) and anti-human TLR3 mAb (TLR3.7) were obtained in our laboratory (11). Anti-human TLR4 mAb (HTA125) was a gift from Dr. K. Miyake (University of Tokyo, Tokyo, Japan) (26). Anti-TLR3 mAb against synthetic peptide of human TLR3 was purchased from BioCarta (San Diego, CA). Anti-CD4, anti-CD45RA, and anti-CD86 mAbs were purchased from Ancell (Bayport, MN). Anti-CD1a, anti-CD2, anti-CD83, and anti-HLA-DR were from Immunotech (Marseille, France), anti-CD64 and anti-CD123 (IL-3Rα) were from BD PharMingen (San Diego, CA), anti-CD8 was from Nichirei (Tokyo, Japan), and anti-CD33 and anti-CD11c were from BD Biosciences (San Jose, CA).

Preparation of viral dsRNA

Primate rotavirus strains were propagated in the monkey kidney cell line MA-104 as previously described (27). dsRNA of rotavirus (SA-11 strain) was prepared from viral infected-MA104 cells by using TRIzol LS (Invitrogen), and 11 segments of dsRNA were checked by RNA-PAGE as described (28). The dsRNA preparation was treated with polymyxin B and its function was assessed using monocyte-derived iDCs and human fibroblast cell line, MRC-5.

Flow cytometry

Cells were incubated with the indicated mAbs (1 μg) together with human IgG (10 μg) for 30 min at 4°C in FACS buffer (Dulbecco's PBS 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, San

Clemente, CA) was added and further incubated for 30 min at 4°C. For intracellular staining, cells were pretreated with permeabilizing solution (BD Biosciences) for 10 min at room temperature, washed once with Dulbecco's PBS and then incubated with mAbs (1 μg) for 30 min at room temperature. After washing, cells were reacted with FITC-labeled secondary Ab at room temperature for 30 min. Goat serum (10%) was added to both reaction mixtures to prevent nonspecific binding. The cells were then analyzed on a FACSCalibur (BD Biosciences).

Immunoprecipitation

Ba/F3 cells stably expressing Flag-tagged human TLR3, HEK293 cells transiently expressing Flag-tagged human TLR3 (11), and HeLa cells endogenously expressing TLR3 were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 25 mM iodoacetamide, and 2 mM PMSF). TLR3 was immunoprecipitated with anti-Flag mAb (M2) or anti-TLR3 mAb (TLR3.7) and subjected to SDS-PAGE (7.5%) under reducing conditions, followed by immunoblotting with M2 anti-Flag mAb (Sigma-Aldrich) or anti-TLR3 mAb (IM-GENEX, San Diego, CA). Mouse IgG1 was used as a negative-control for immunoprecipitation.

To detect TLR3 protein expressed in monocyte-derived iDCs, 4.5×10^7 cells were washed three times in PBS and resuspended in hypotonic buffer (10 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 20 mM HEPES, pH 7.4) on ice for 20 min. Cells were lysed by Dounce homogenizer (50 strokes) and sequentially centrifuged at $600 \times g$ to pellet nuclei, and $10,000 \times g$ to pellet mitochondria and lysosome. The pellet ($10,000 \times g$) was lysed in the lysis buffer. TLR3 protein in the lysate was immunoprecipitated as previously described.

Cytokine assays

Monocyte-derived iDCs in 24-well plates (6.6×10^5 /ml), or BDCA-1⁺ myeloid blood DCs in 96-well round bottom plates (3×10^5 /ml) were stimulated with LPS (100 ng/ml), MALP-2 (100 nM), or poly(I:C) (50 μg/ml) for 4 to 24 h. BDCA-4⁺ plasmacytoid DCs in round bottom 96-well plates (1.25×10^5 /ml) were stimulated with LPS, MALP-2, poly(I:C), or CpG ODN 2006 (2 μM) in the presence of 500 U/ml of rhIL-3 (PeproTech). For the Ab inhibition assay, monocyte-derived iDCs were pretreated with 20 μg/ml of anti-TLR2 mAb (TLR2.45) or anti-TLR3 mAb (TLR3.7) for 30 min at 37°C, then stimulated with polymyxin B-treated poly(I:C) (0, 5, 10, 20 μg/ml) for 24 h. The levels of IFN-α, IFN-β, and IL-12p70 in the culture supernatants were measured by ELISA:IFN-α (BioSource International, Camarillo, CA), IFN-β (TFB, Tokyo, Japan), and IL-12p70 (Amersham Pharmacia Biotech).

MLR assay

Practical method was described in a previous report (29). Briefly, monocyte-derived iDCs were cultured with or without poly(I:C) (5 and 20 μg/ml) in the presence of GM-CSF (500 U/ml) for 24 h. The cells were irradiated (3000 rad) and cultured for 4 days with 1×10^5 allogeneic lymphocytes in 96-well plates in 100 μl RPMI 1640 containing 10% FCS. The lymphocyte proliferation was determined using a cell proliferation assay kit (CellTiter 96; Promega, Madison, WI).

Immunofluorescence microscopy

Monocytes, monocyte-derived iDCs, BDCA-1⁺ myeloid blood DCs, or BDCA-4⁺ plasmacytoid DCs were adhered to glass slides by Cytospin (Thermo Shandon, Cheshire, U.K.). The adhered cells were fixed for 30 min with 3% formaldehyde in PBS and were permeabilized with 0.5% saponin, 1% BSA/PBS for 30 min, washed four times with PBS. After soaking in 1% BSA/PBS, cells were treated for 1 h at room temperature with anti-TLR3 mAb (TLR3.7) or normal mouse IgG (20 μg/ml) (Sigma-Aldrich) in 1% BSA/PBS. Cells were then washed with 1% BSA/PBS and treated for 30 min at room temperature with rhodamine B-conjugated goat anti-mouse IgG (BioSource International) (1:100) in PBS containing 10% (w/v) Block Ace (Yukijirushi, Sapporo, Japan). The stained cells were visualized at $\times 60$ magnification under a FLUOVUEVIEW FV300 (Olympus, Melville, NY). Images were captured using the attached computer software, FLUOVUEVIEW.

Immunoelectron microscopy

For immunoelectron microscopy, the pre-embedding silver enhancement immunogold method was used with a slight modification (30). Briefly, a pellet of the cells was fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 2 h. Cryosections (6 μm in thickness) were

made and reacted with Abs, followed by incubation with colloidal gold-conjugated (diameter 1.4 nm) secondary Abs. The gold labeling was intensified using a silver enhancement kit, HQ silver (Nanoprobes).

Results

Characterization of anti-TLR3 mAb, TLR3.7

First, we examined whether the mAb TLR3.7 can immunoprecipitate human TLR3 expressed in a variety of cells. This mAb recognizes the extracellular portion of native TLR3, but fails to react with a denatured-form of TLR3 on blots (11). Flag-tagged TLR3 in lysates of Ba/F3 and HEK293 transfectants and endogenous TLR3 in HeLa cells were pulled down with TLR3.7 and resolved on SDS-PAGE followed by immunoblotting. The blots were probed with anti-Flag mAb or anti-human TLR3 peptide Ab (Fig. 1). TLR3 proteins were detected in the lanes using the specific mAb TLR3.7, suggesting that TLR3.7 can catch up TLR3 in cell lysates.

Expression of TLR3 in monocyte-derived iDCs

The protein expression levels of human TLR2, TLR3, and TLR4 in monocyte-derived iDCs were analyzed by flow cytometry using the characterized mAbs against human TLR2, TLR3, and TLR4. The surface marker profile of the iDCs prepared is shown in Fig. 2A. Several reports demonstrated that TLR2, TLR3, and TLR4 are expressed in monocyte-derived iDCs at the message levels (7, 9). At the protein level, TLR2 and TLR4 were marginally detected by cell surface staining, while TLR3 was detected only by intracellular staining (Fig. 2B). Expressions of the mRNAs of TLR2, TLR3, and TLR4 in these cells were confirmed by RT-PCR (Fig. 2C).

Differentiation of monocytes into iDCs by GM-CSF and IL-4 induced TLR3 protein on day 1, the expression level of TLR3 was increased on day 3, and sustained up to day 6 in both protein and message levels (Fig. 3, A and B). The expression level and localization of TLR3 did not change during DC maturation induced by LPS or MALP-2, while its intracellular expression level was slightly up-regulated by stimulation with TLR3 ligand, poly(I:C) (Fig. 3C). The TLR3 mRNA up-regulation by poly(I:C) was confirmed by RT-PCR (Fig. 3D).

Expression of TLR3 in human blood DCs

We next examined the TLR3 expression in human blood DCs, CD11c⁺ blood DCs and the second type of pre-DC2 (previously

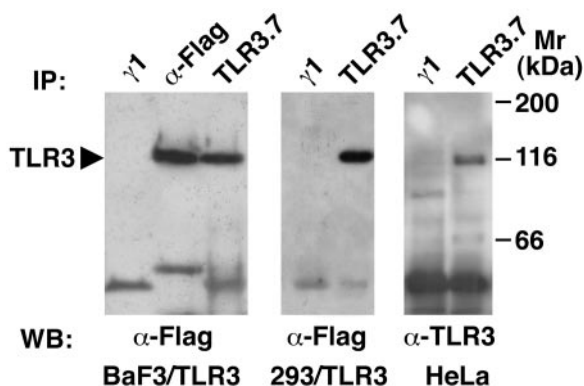


FIGURE 1. Immunoprecipitation of TLR3 protein in lysates of transfectants or HeLa cells with anti-TLR3 mAb. Ba/F3 cells or HEK293 cells expressing Flag-tagged human TLR3, or HeLa cells were lysed in lysis buffer. TLR3 protein was immunoprecipitated with nonimmune mouse IgG ($\gamma 1$), anti-Flag mAb (α -Flag) or anti-TLR3 mAb (TLR3.7), followed by Western blotting with anti-Flag mAb (α -Flag) or anti-TLR3 peptide Ab (α -TLR3). Arrowhead indicates TLR3 with molecular mass of 116 kDa.

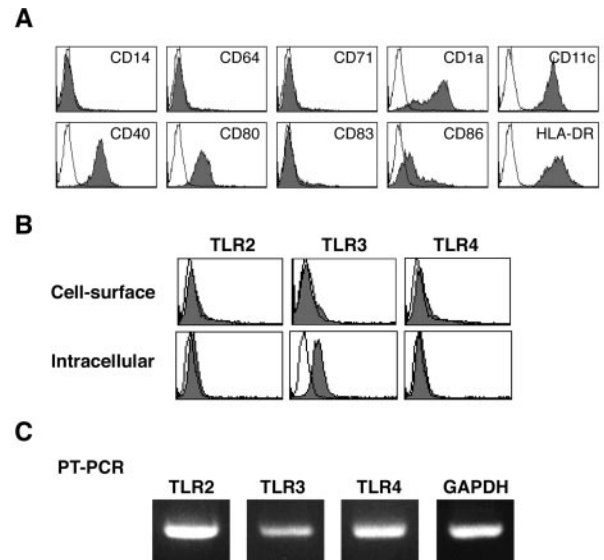


FIGURE 2. Expression profiles of TLR2, TLR3, and TLR4 in monocyte-derived iDCs. Cell surface phenotype (A) and expression levels of TLR2, TLR3, and TLR4 (B) in monocyte-derived iDCs were assessed by flow cytometry using specific mAb including TLR2.45 (anti-TLR2), TLR3.7 (anti-TLR3), and HTA125 (anti-TLR4) (shaded histogram). Open histogram represents cells labeled with isotype-matched control Abs. B, Cell surface staining (upper panel) and intracellular staining (lower panel) are shown. C, RT-PCR analysis of TLR2, TLR3, and TLR4 mRNA expression in monocyte-derived iDCs is shown. GAPDH mRNA was measured as a control.

known as plasmacytoid DC precursors), which were recently identified as natural type I IFN-producing cells in human blood (17). CD11c⁺ blood DCs were isolated from PBMCs using BDCA-1 (CD1c) cell isolation kit. The CD1c Ag is expressed on a major subpopulation of myeloid blood DCs in human blood (31, 32). The freshly isolated CD1c (BDCA-1⁺) myeloid blood DCs were CD1a⁻, CD4⁺, CD11c⁺, CD123^{dim}, CD33⁺, CD64⁺, CD80⁻, CD83⁻, CD86^{low}, and HLA-DR^{high}, the phenotype matching those in previous reports (31, 32) (Fig. 4A). The cells expressed TLR2, TLR3, and TLR4 similar to monocyte-derived iDCs, but cell surface expression level of TLR2 was higher than that of monocyte-derived iDCs (Fig. 4A).

In contrast, BDCA-4⁺ plasmacytoid blood DCs were used as pre-DC2, because BDCA-4 Ag was shown to specifically express on CD11c⁻CD123^{bright} plasmacytoid DCs (31, 32). The freshly isolated BDCA-4⁺ blood DCs were CD4⁺HLA-DR⁺CD45RA⁺CD123(IL-3R α)⁺CD11c⁻; their surface phenotype was identical with that of pre-DC2 (Fig. 4B). They also lacked CD1a, CD2, CD8, and CD64. Although the mRNA of TLR3 was not detectable in BDCA-4⁺ plasmacytoid DCs (Fig. 4B), TLR3 signal was detected by intracellular staining (Fig. 4Bb). One possibility of this protein-mRNA discrepancy is that anti-TLR3 mAb cross-reacted with an intracellular molecule other than TLR3 in BDCA-4⁺ cells. To confirm this possibility and where TLR3 is localized in myeloid DCs, immunofluorescent staining with anti-TLR3 mAb was performed and analyzed by confocal microscopy. As shown in Fig. 5A, TLR3 resided in the subcellular compartment but not on the plasma membrane in monocyte-derived iDCs and CD11c⁺ blood DCs. In addition, a spot-like fluorescent signal was detected near the nucleus in myeloid DCs and BDCA-4⁺ plasmacytoid DCs (Fig. 5A), which nearly merged with centrosome defined by γ -tubulin (data not shown). Spot-like staining was not observed in monocytes (Figs. 3A and 5A), suggesting that the anti-TLR3 mAb

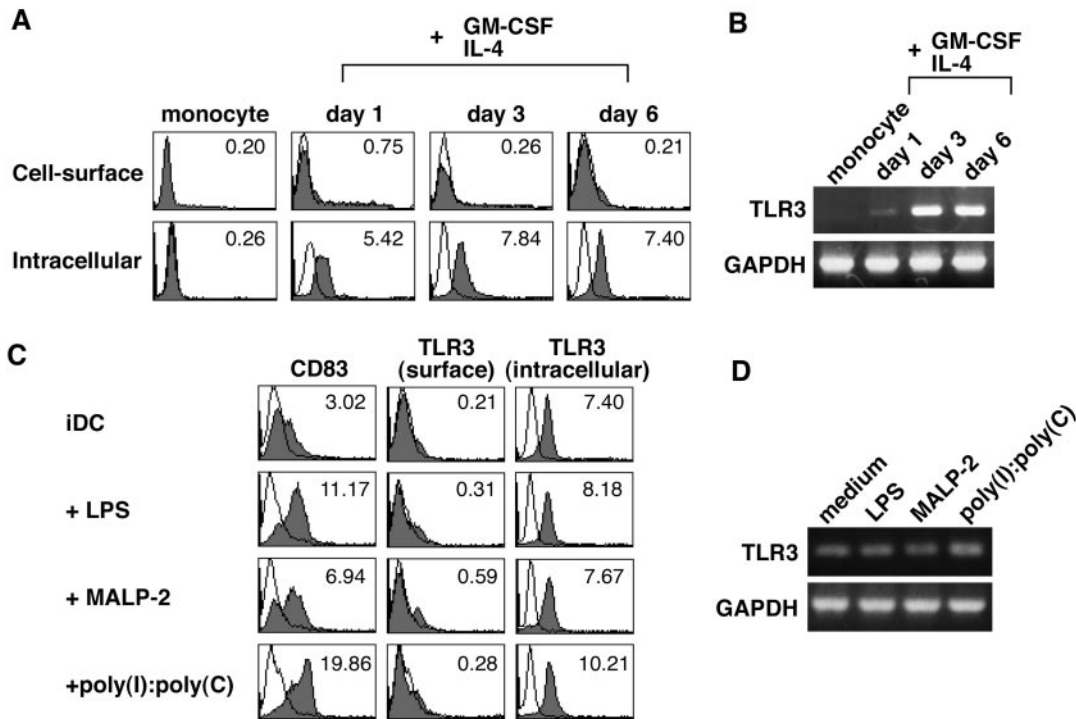


FIGURE 3. Expression of TLR3 during iDC differentiation. *A*, Freshly isolated monocytes were cultured for 6 days in the presence of GM-CSF and IL-4. At timed intervals, intact or permeabilized cells were stained with anti-TLR3 mAb and FITC-labeled secondary Ab, then analyzed by FACS (shaded histogram). Open histogram represents cells labeled with control mouse IgG1. *Inset* values indicate the mean fluorescent intensities specific for the anti-TLR3 mAb. *B*, RT-PCR analysis of TLR3 mRNA expression was performed using RNA preparations isolated from monocytes cultured in the presence of GM-CSF and IL-4 for indicated times. Amplification of the GAPDH gene from the same samples confirmed the equivalence of the amounts of cDNA. *C*, TLR3 expression in matured DCs. iDCs were stimulated with LPS (10 ng/ml), MALP-2 (100 nM), or poly(I):poly(C) (12.5 μ g/ml) for 40 h to obtain mature DCs (mDCs). Expressions of CD83 (DC maturation marker) and TLR3 in mDCs were analyzed by FACS. Poly(I):poly(C) stimulation up-regulated intracellular TLR3 expression in iDCs. *Inset* values indicate the mean fluorescence intensities specific for the anti-CD83 or anti-TLR3 mAb. *D*, TLR3 mRNA expression was up-regulated by stimulation of iDCs with poly(I):poly(C). Total RNA was prepared from iDCs stimulated with medium alone, LPS, MALP-2, or poly(I):poly(C) for 24 h, and subjected to RT-PCR analysis of TLR3 mRNA.

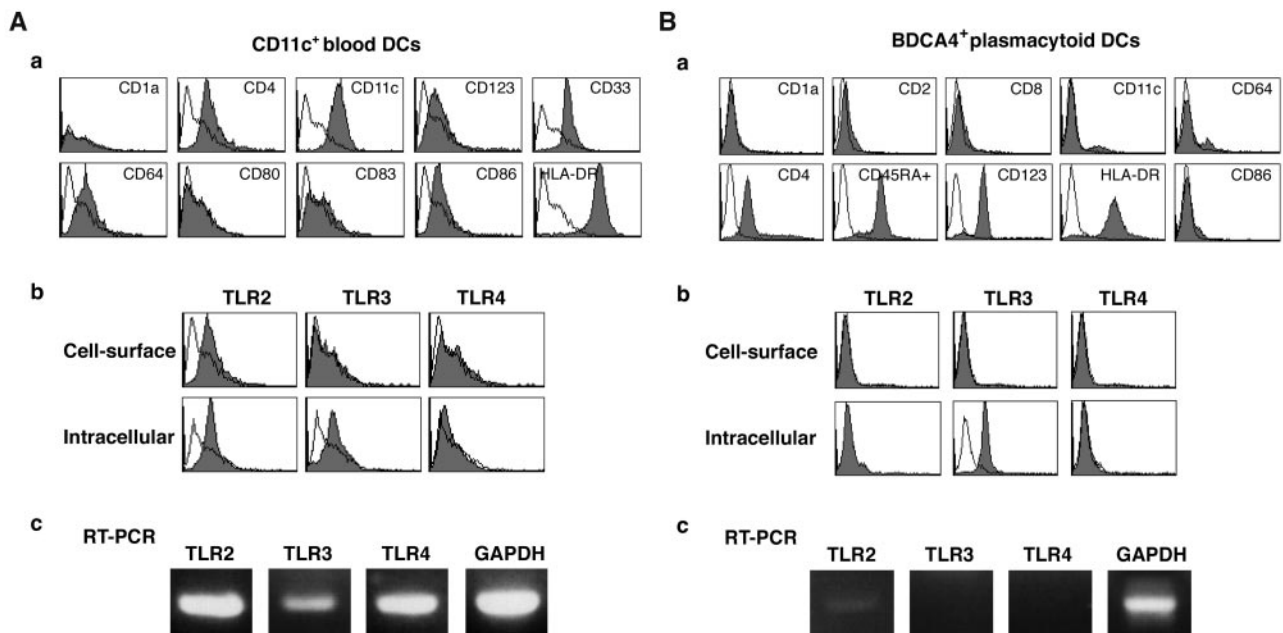


FIGURE 4. Expression profiles of TLR2, TLR3, and TLR4 in human blood DC subsets. Cell surface phenotype (*a*) and expression levels of TLR2, TLR3, and TLR4 (*b*) in BDCA-1⁺ myeloid blood DCs (*A*) and BDCA-4⁺ plasmacytoid blood DCs (*B*) were assessed by flow cytometry using specific mAbs. Open histogram represents cells stained with isotype-matched control Abs. The data shown are representative of four experiments. RT-PCR analysis of TLR2, TLR3, and TLR4 mRNA expression (*c*) in BDCA-1⁺ myeloid blood DCs and BDCA-4⁺ plasmacytoid blood DCs. GAPDH mRNA was measured as a control (*c*). In plasmacytoid DCs, the protein-mRNA discrepancy of TLR3 expression was observed (see *Results*).

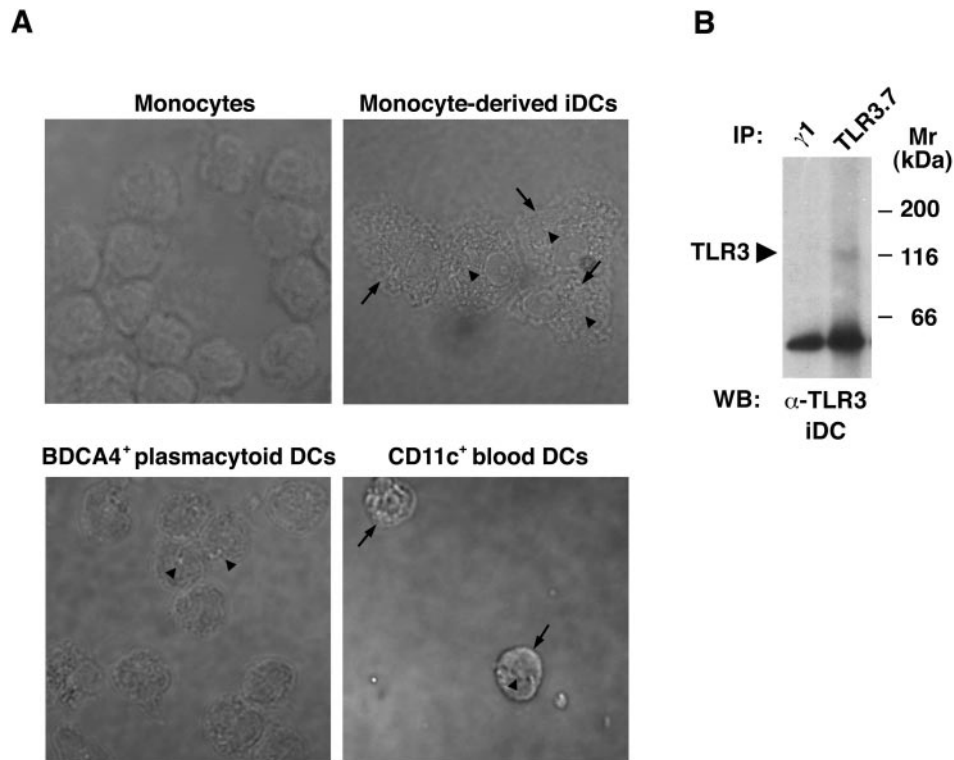


FIGURE 5. Intracellular localization of human TLR3 in monocyte-derived iDCs and CD11c⁺ myeloid blood DCs. **A**, Immunofluorescent analysis of TLR3 expression in human DC subsets is shown. Monocytes, monocyte-derived iDCs, BDCA-1⁺ myeloid blood DCs, and BDCA-4⁺ plasmacytoid DCs were permeabilized and stained with anti-TLR3 mAb or control mouse IgG (data not shown) and then with rhodamine B-conjugated secondary Ab. The stained cells were visualized at $\times 60$ magnification under a FLUOVIEW FV300 (Olympus). TLR3 localizes to subcellular compartment in monocyte-derived iDCs and CD11c⁺ blood DCs. Arrows indicate TLR3 localized to intracellular compartments. Arrowheads indicate a putative centrosomal protein shared an epitope with TLR3. Monocytes do not express TLR3. **B**, Immunoprecipitation of TLR3 protein in lysates of mitochondria/lysosome-enriched fraction of monocyte-derived iDCs with anti-TLR3 mAb is shown. The mitochondria/lysosome-enriched 10,000 \times g pellet was lysed in the lysis buffer. TLR3 protein was immunoprecipitated with nonimmune mouse IgG ($\gamma 1$) or anti-TLR3 mAb (TLR3.7), followed by Western blotting with anti-TLR3 peptide Ab (α -TLR3). Arrowhead indicates TLR3 with molecular mass of 116 kDa.

(TLR3.7) recognizes centrosomal protein that shared an epitope with TLR3. This unidentified protein was expressed in myeloid DCs and plasmacytoid DCs but not in peripheral blood monocytes. Endogenous TLR3 protein was also detected in monocyte-derived iDCs by immunoprecipitation/immunoblotting analysis (Fig. 5B).

dsRNA stimulates monocyte-derived iDCs to produce cytokines and up-regulate costimulatory molecules

We next tested whether these DC subsets produced IFN- α/β and IL-12p70 in response to MALP-2 (a TLR2 ligand), LPS, poly(I:C), or CpG ODN 2006 (a TLR9 ligand). Monocyte-derived iDCs produced IL-12p70 and IFN- α/β in response to poly(I:C) or LPS, while BDCA-4⁺ plasmacytoid DCs produced IFN- α in response to CpG ODN (Fig. 6A). Similar to monocyte-derived iDCs, BDCA-1⁺ myeloid DCs produced IFN- β in response to poly(I:C), but its level was relatively low (data not shown). TLR2 signaling did not induce any IL-12p70, IFN- α , or IFN- β in either myeloid or BDCA-4⁺ DCs. Although a previous report showed that CpG ODN 2006 did not induce pre-DC2 to produce IFN- α (33), freshly isolated BDCA-4⁺ plasmacytoid DCs induced large amounts of IFN- α in response to ODN 2006, suggesting that there may be some differences between pre-DC2 and BDCA-4⁺ plasmacytoid DCs.

Consistent with the effect on DC maturation (Fig. 3C) and cytokine production, poly(I:C) increased the capacity of monocyte-derived iDCs to stimulate a proliferative response by allogenic lymphocytes (Fig. 6B). In addition, dsRNA isolated from rotavirus activated iDCs resulting in up-regulation of CD86 and HLA class

II molecules (Fig. 6C). These results suggest that virus-derived dsRNA as well as poly(I:C) participates in antiviral immunity by inducing cytokine production and DC maturation.

TLR3 does not act on the cell surface in DCs

We next tested whether TLR3 expressed in the myeloid lineage of DCs is competent to dsRNA recognition. Interestingly, function-blocking anti-TLR3 mAb could not inhibit poly(I:C)-induced IFN- β production by monocyte-derived iDCs (Fig. 7A). Furthermore, long-term poly(I:C) stimulation was required to induce IFN- β and IL-12p70 production in iDCs (Fig. 7B). In short-term stimulation (4 h), IFN- β protein was below the detection limit in the culture supernatants of iDCs, which is completely different from fibroblasts expressing cell surface TLR3 (11). As TLR3 localizes intracellular compartment in myeloid DCs, we next examined whether endosomal maturation is required in poly(I:C)-mediated cytokine production similar to CpG DNA-mediated TLR9 signaling (34). In the presence of 5 μ g/ml of endosomal acidification inhibitor, chloroquine, which dose is sufficient to inhibit CpG DNA-mediated cytokine production in B cells and monocytes, poly(I:C)-mediated IL-12p70 production by iDCs was markedly inhibited (Fig. 7C). Under the same conditions, action of LPS was not inhibited when judged by the level of IL-12p70 (Fig. 7C). Thus, dsRNA should be internalized into the acidified intracellular compartment in iDCs, in which TLR3 may reside for encounter with its ligands.

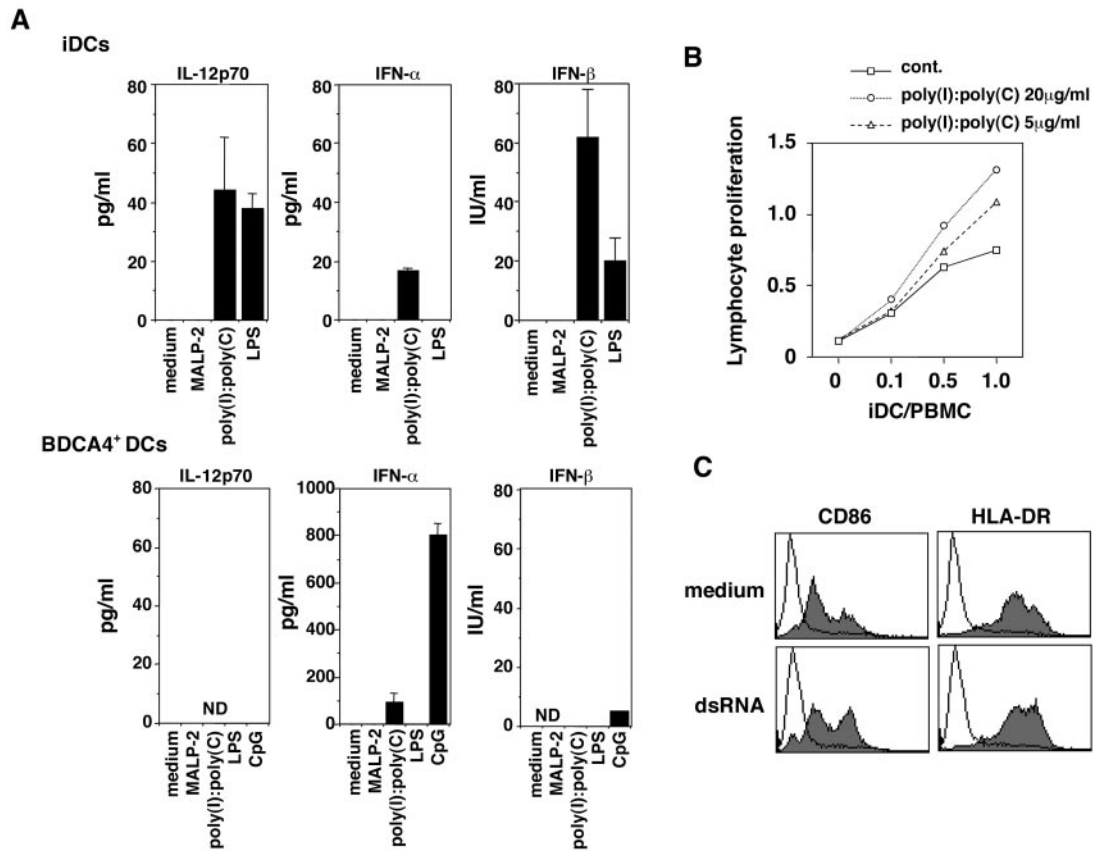


FIGURE 6. DC maturation was induced by dsRNA. *A*, Cytokine production by monocyte-derived iDCs and BDCA-4⁺ plasmacytoid DCs stimulated with a distinct ligand for TLRs. Cells were stimulated with 100 ng/ml LPS (TLR4 ligand), 100 nM MALP-2 (TLR2/TLR6 ligand), 50 μ g/ml poly(I:C) (TLR3 ligand), or 2 μ M ODN 2006 (TLR9 ligand) for 24 h. The concentrations of IFN- α/β and IL-12p70 in the culture supernatants were measured by ELISA. The data shown are representative of four independent experiments, each performed in duplicate. ND, not detected. *B*, MLR analysis using poly(I:C)-stimulated monocyte-derived iDCs is shown. iDCs were treated with indicated amounts of poly(I:C), and after washing, various numbers of irradiated DCs were incubated with freshly isolated PBMCs from a single individual. Four days later, lymphocyte proliferation was measured by cell proliferation assay kit (Promega). One representative of the four similar experiments is shown. *C*, Viral dsRNA induced DC maturation in monocyte-derived iDCs. iDCs were cultured with medium alone or polymyxin B-treated dsRNA of rotavirus (1 μ g/ml) for 40 h. The up-regulation of CD86 and HLA-DR expression was assessed by flow cytometry. IL-12p70 was also induced by rotavirus dsRNA (not shown).

TLR3 localizes to multivesicular bodies (MVBs) in Ba/F3 cells expressing human TLR3

The subcellular compartment that TLR3 localizes to was further analyzed by immunoelectron microscopy using stable Ba/F3 transfectants expressing human TLR3. FACS analysis showed that TLR3 was predominantly present inside the cells and slightly expressed on the cell surface (Fig. 8A). Anti-Flag mAb detected TLR3 after the cells were permeabilized, because Flag tag was attached to the C terminus of TLR3. By observation with electron microscopy, the Ba/F3 cells were found to possess relatively large vacuoles containing multiple vesicles in the lumen, called MVBs. MVBs are late endosomal compartments situated in the endocytic route between early endosomes and lysosomes (35–37). In APCs such as B cells and DCs, MHC class II is stored intracellularly at the internal vesicles of MVBs (38–40). In the Ba/F3 cells stably expressing human TLR3, TLR3 molecules were found predominantly on the internal membrane of MVBs by immunoelectron microscopy with anti-TLR3 or anti-Flag mAb and then gold-labeled secondary Ab (Fig. 8B). A small number of TLR3 molecules were detected on the limiting membrane of MVBs and on the plasma membrane (Fig. 8B). Although no drastic change of TLR3 expression both on the plasma membrane and intracellular were seen after 24-h stimulation with poly(I:C), cell surface expression of TLR3 was slightly increased through TLR3 signaling (Fig. 8C).

These results suggest that in Ba/F3 cells, endocytic trafficking activity is somewhat increased by poly(I:C) stimulation, but most TLR3 molecules are largely stored intracellularly like monocyte-derived iDCs.

Discussion

In this study, we first analyzed the localization of TLR3 in human DC subsets. Unlike fibroblasts, monocyte-derived iDCs express TLR3 intracellularly but not on the cell surface. This DC subset produced IFN- α/β and IL-12p70 in response to poly(I:C), a synthetic ligand for TLR3. The Ab-blocking research (Fig. 7A) suggests that TLR3 does not act on the cell surface in iDCs. Similar to monocyte-derived iDCs, CD11c⁺ blood DCs also expressed TLR3 inside the cells and produced IFN- β in response to poly(I:C). These results suggest that myeloid-lineage DCs express TLR3 in their cytoplasmic compartment, the distribution profiles of which differ from that of fibroblasts expressing TLR3 on the cell surface.

BDCA-4⁺ plasmacytoid DCs, on the other hand, do not express TLR3 either inside or outside the cells consistent with previous reports (21, 22). BDCA-4 Ag is a novel human DC marker, which is expressed on CD11c⁻CD123^{bright} plasmacytoid blood DCs and also appeared on monocyte-derived and CD34⁺ cell-derived CD1a⁺ DCs (31, 32). Interestingly, the anti-TLR3 mAb (TLR3.7) cross-reacts with an intracellular molecule expressed in BDCA-4⁺

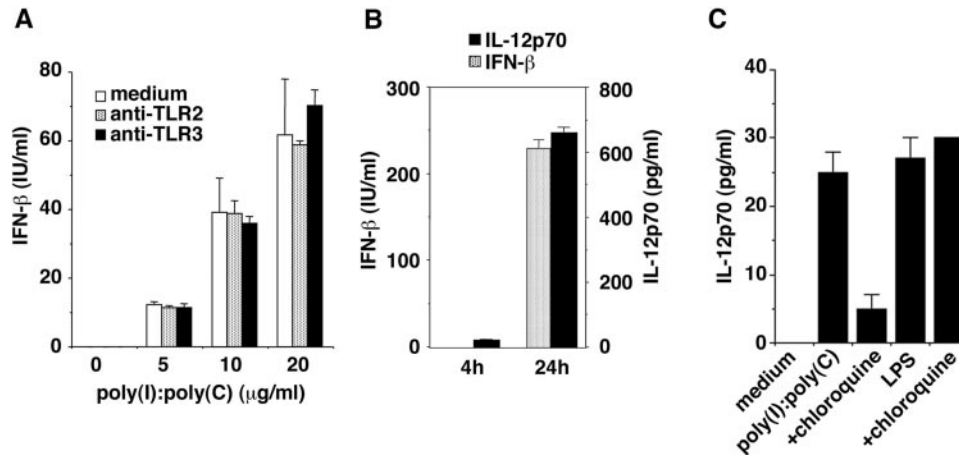


FIGURE 7. TLR3 does not act on the cell surface. *A*, Failure of anti-TLR3 mAb to inhibit poly(I:C)-induced IFN- β production by monocyte-derived iDCs. iDCs were pretreated with medium alone, 20 μ g/ml of anti-TLR2 mAb (TLR2.45) or anti-TLR3 mAb (TLR3.7) for 30 min at 37°C, then stimulated with poly(I:C) (0, 5, 10, and 20 μ g/ml) for 24 h. The levels of IFN- β in the culture supernatants were measured by ELISA. *B*, Long-term incubation with poly(I:C) is required for cytokine production by iDCs. iDCs were stimulated with 50 μ g/ml poly(I:C) for indicated times. The concentrations of IFN- β and IL-12p70 in the culture supernatants were measured by ELISA. *C*, Blockade of poly(I:C)-induced cytokine production by inhibitor of endosomal acidification in monocyte-derived iDCs. iDCs (10^6 cells/ml) were cultured with or without chloroquine (5 μ g/ml) for 2 h and then stimulated with poly(I:C) (50 μ g/ml) or LPS (100 ng/ml) for 5 h. ELISA for IL-12p70 was performed on the supernatants.

cells and myeloid DCs. This cross-reactive Ag shares an epitope with TLR3 and nearly merged with the centrosome marker, suggesting that this is a centrosomal protein.

In myeloid-lineage DCs, TLR3 appears to localize to endosomal compartments (Fig. 5), but any organelle marker we tested and MHC class II molecule did not colocalize with TLR3 (K. Funami, M. Matsumoto, A. Yamamoto, and T. Seya, unpublished observations). Notably, TLR3 was shown to specifically localize to MVBs in a stable transfectant Ba/F3 cell, which is an IL-3-dependent murine B cell line (Fig. 8). MVBs are late endosomal compartments situated in the endocytic trafficking pathways, and are responsible for both the biosynthetic delivery of lysosomal proteins and the down-regulation of activated cell surface receptors (35–37). They are 300–500 nm vacuoles with numerous membrane vesicles in their lumens. Internal vesicles of MVBs are generated by inward budding of the limiting membrane (36). In APCs such as B cells and DCs, MHC class II is stored intracellularly in MVBs, referred to as MHC class II-enriched compartments or a class II-containing vesicle, which is a specialized site for peptide loading (38–40). Stimulation of DCs with living bacteria or bacterial components such as LPS results in the reorganization of MVBs and increased surface expression of MHC class II at the plasma membrane (40). In Ba/F3 cells, TLR3 molecules resided in the internal membrane of MVBs, and small numbers of TLR3 were detected on the limiting membrane of MVBs and on the plasma membrane (Fig. 8B). However, no drastic change of TLR3 expression was seen either on the plasma membrane or inside the cell after 24-h stimulation with poly(I:C) (Fig. 8C). Thus, TLR3 molecules are stored in intracellular compartments in Ba/F3 cells like monocyte-derived iDCs.

The regulation of human TLR3 expression is different from that of murine TLR3 (41). LPS did not up-regulate the TLR3 expression in human monocyte-derived iDCs, while poly(I:C) induced both murine and human TLR3 expressions in DCs (Fig. 3). A previous report showed that human TLR3 mRNA is up-regulated by viral infection or treatment of macrophages with type I IFN (42). Poly(I:C) induces IFN- α/β secretion by murine DCs and the released IFN- α/β acts in an autocrine manner through the IFN- α/β receptor to induce TLR3 expression in DCs (43). In either case,

TLR3 expression is up-regulated intracellularly. In this study, human TLR3 was undetectable on the cell surface after the treatment of monocyte-derived iDCs with poly(I:C) or IFN- β (Fig. 3C, data not shown). Although the mechanisms by which TLR3 is enriched into the vacuoles are under investigation, the mode of dsRNA-TLR3 interaction in myeloid DCs should differ from that in fibroblasts.

In this study, we first demonstrated that dsRNA of viral origin activates DCs in a similar manner to poly(I:C). Although TLR3 does not present on the cell surface in DCs, exogenously added dsRNA activates the cells to produce IFN- α/β and IL-12p70, suggesting that after internalization, dsRNA encounters intracellular TLR3 present in the subcellular compartments and activated TLR3 transduces signals inside the cells. Supporting this issue is that 1) an endosomal acidification inhibitor, chloroquine, inhibited IL-12p70 liberation from iDCs by poly(I:C), 2) long-term incubation with poly(I:C) was required for IFN- β and IL-12p70 induction (Fig. 7). Because we just extrinsically added poly(I:C) or dsRNA to cells without using transfection reagents, no direct association between poly(I:C) and cytoplasmic proteins could be verified.

A possibility might have remained, however, that poly(I:C) internalized could activate protein kinase R (PKR) as suggested in mouse embryonic fibroblasts. PKR is a cytoplasmic protein and has been characterized as a major intracellular RNA-recognition molecule inducing antiviral cellular response (44). Nevertheless, PKR knockout mice and cells showed no severe phenotype, which suggested the presence of an additional protection strategy against viral infection (45). Based on these results, together with our present finding indicating that dsRNA is taken up into the endosomal compartments, we hold that the viral dsRNA recognition system barely involves cytoplasmic PKR in DCs, at least under our relevant conditions. Our findings might provide new insight into the physiological antiviral DC functions in conjunction with reported knowledge on the recognition system of dsRNA (46). Further study including where TLR3 encounters dsRNA and how signals are transduced in DCs will be needed to consolidate the importance of the TLR3-mediated antiviral DC functions.

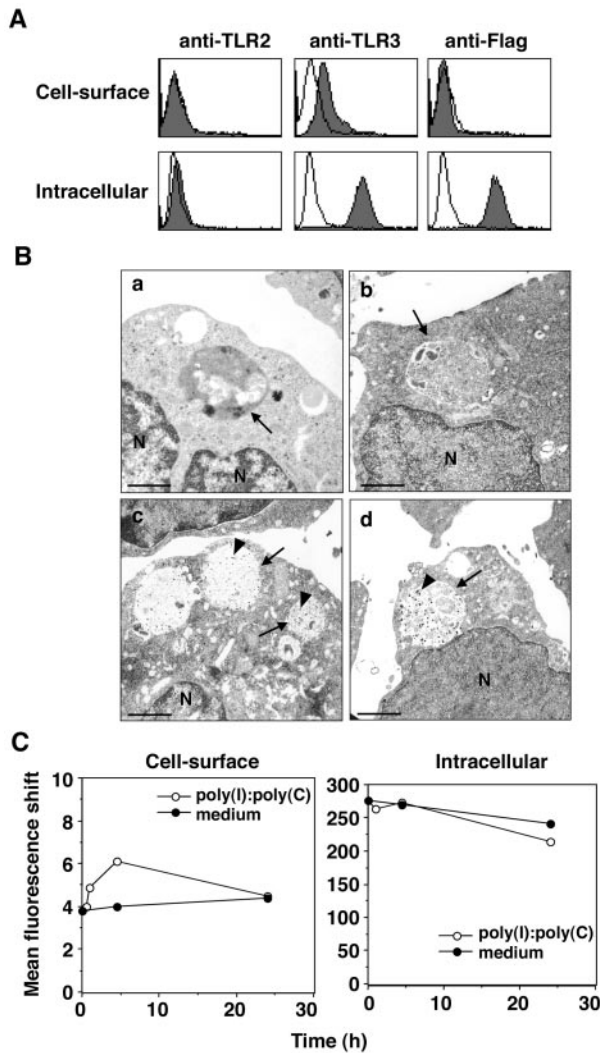


FIGURE 8. TLR3 localizes on the intravesicular membranes of MVBs in the murine B cell line Ba/F3 that stably expresses human TLR3. *A*, FACS analysis of TLR3 expression in Ba/F3 cells expressing Flag-tagged human TLR3 using anti-TLR3 or anti-Flag mAbs is shown. Anti-Flag mAb detected TLR3 after the cells were permeabilized, because the Flag epitope was attached to the C terminus of TLR3. Anti-TLR2 mAb was used as a control. Open histogram represents cells stained with mouse IgG1. *B*, Immunoelectron micrograph of an ultrathin cryosection of Ba/F3 cells expressing Flag-tagged human TLR3. Electron micrograph of Ba/F3 cells (*a*) shows a large MVB (arrow) in Ba/F3 cells. Ba/F3 cells expressing Flag-tagged human TLR3 were labeled with control mIgG1 (*b*), anti-TLR3 mAb (*c*), or anti-Flag mAb (*d*) and gold-conjugated secondary Ab, and processed for silver enhancement. TLR3 was present on the intravesicular membranes of MVBs (arrowhead) (*c* and *d*). Arrows indicate MVBs. The nuclei (N) are marked, bar, 1 μ m. *C*, No marked change of TLR3 expression was observed by function of poly(I:C) both on the plasma membrane and inside the Ba/F3 cells expressing Flag-tagged human TLR3. Cells were stimulated with 10 μ g/ml poly(I:C) for indicated times. The TLR3 expression levels on the cell surface and in the cells were assessed by FACS.

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References

- Medzhitov, R., and C. A. Janeway, Jr. 1997. Innate immunity: the virtues of non-clonal system of recognition. *Cell* 91:295.
- Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406:782.
- Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immun.* 2:675.
- Underhill, D. M., and A. Ozinsky. 2002. Toll-like receptors: key mediators of microbe detection. *Curr. Opin. Immunol.* 14:103.
- Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388:394.
- Rock, F. L., G. Hardiman, J. C. Timans, R. A. Kastelein, and J. F. A. Bazan. 1998. A family of human receptors structurally related to *Drosophila* Toll. *Proc. Natl. Acad. Sci. USA* 95:588.
- Muzio, M., D. Bosisio, N. Polentarutti, G. D'amico, A. Stoppacciaro, R. Mancinelli, C. van't Veer, G. Penton-Rol, L. Ruco, P. Allavena, and A. Mantovani. 2000. Differential expression and regulation of Toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J. Immunol.* 164:5998.
- Cario, E., and D. K. Podolsky. 2000. Differential alteration in intestinal epithelial cell expression of Toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect. Immun.* 68:7010.
- Visintin, A., A. Mazzoni, J. H. Spitzer, D. H. Wyllie, S. K. Dower, and D. M. Segal. 2001. Regulation of Toll-like receptors in human monocytes and dendritic cells. *J. Immunol.* 166:249.
- Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of double-stranded RNA and activation of NF- κ B by Toll-like receptor 3. *Nature* 413:732.
- Matsumoto, M., S. Kikkawa, M. Kohase, K. Miyake, and T. Seya. 2002. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem. Biophys. Res. Commun.* 293:1364.
- Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740.
- Yamamoto, M., S. Sato, H. Hemmi, H. Sanjo, S. Uematsu, T. Kaisho, K. Hoshino, O. Takeuchi, M. Kobayashi, T. Fujita, K. Takeda, and S. Akira. 2002. Essential role for TIRAP in activation of the signaling cascade shared by TLR2 and TLR4. *Nature* 420:324.
- Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya. 2003. TICAM-1, an adapter molecule that participates in Toll-like receptor 3-mediated interferon- β induction. *Nat. Immun.* 4:161.
- Muller, U., U. Steinhoff, L. F. L. Reis, S. Hemmi, J. Pavlovic, R. M. Zinkernagel, and M. Agut. 1994. Functional role of type I and type II interferons in antiviral defense. *Science* 264:1918.
- Biron, C. A. 2001. Interferons α and β as immune regulators: a new look. *Immunity* 14:661.
- Siegel, F. P., N. Kadowaki, M. Shodell, P. A. Fitzgerald-Bocarsly, K. Shah, S. Ho, S. Antonenko, and Y. J. Liu. 1999. The nature of the principal type 1 interferon-producing cells in human blood. *Science* 284:1835.
- Blom, B., S. Ho, S. Antonenko, J. Y. N. Lau, and Y. J. Liu. 2000. Generation of interferon α -producing dendritic cell (pre-DC) 2 from human CD34⁺ hematopoietic stem cells. *J. Exp. Med.* 192:1785.
- Verdijk, R. M., T. Mutis, B. Esendam, J. Kamp, C. J. M. Melief, A. Brand, and E. Goulmy. 1999. Polyriboinosinic polyribocytidylic acid (poly(I:C)) induces stable maturation of functionally active human dendritic cells. *J. Immunol.* 163:57.
- Cella, M., M. Salio, Y. Sakakibara, H. Langen, I. Julkunen, and A. Lanzavecchia. 1999. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J. Exp. Med.* 189:821.
- Kadowaki, M., S. Ho, S. Antonenko, R. de Waal Malefyt, R. A. Kastelein, F. Bazan, and Y. J. Liu. 2001. Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. *J. Exp. Med.* 194:863.
- Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of Toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* 168:4531.
- Tsuji, S., M. Matsumoto, O. Takeuchi, S. Akira, I. Azuma, A. Hayashi, K. Toyoshima, and T. Seya. 2000. Maturation of human dendritic cells by cell-wall skeleton of *Mycobacterium bovis* bacillus Calmette-Guérin: involvement of Toll-like receptors. *Infect. Immun.* 68:6883.
- Nishiguchi, M., M. Matsumoto, T. Takao, M. Hoshino, Y. Shimonishi, S. Tsuji, N. A. Begum, O. Takeuchi, S. Akira, K. Toyoshima, and T. Seya. 2001. *Mycoplasma fermentans* lipoprotein M161Ag-induced cell activation is mediated by Toll-like receptor 2: role of N-terminal hydrophobic portion in its multiple functions. *J. Immunol.* 166:2610.
- Takeuchi, O., A. Kaufmann, K. Grote, T. Kawai, K. Hoshino, M. Morr, P. F. Muhlradt, and S. Akira. 2000. Preferentially the R-stereoisomer of the mycoplasma lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a Toll-like receptor 2- and MyD88-dependent signaling pathway. *J. Immunol.* 164:554.
- Shimazu, R., S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto. 1999. MD-2, a molecule that confer lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med.* 189:1777.
- Urasawa, T., S. Urasawa, and K. Taniguchi. 1981. Sequential passage of human rotavirus in MA-104 cells. *Microbiol. Immunol.* 25:1025.

28. Taniguchi, K., S. Urasawa, and T. Urasawa. 1982. Electrophoretic analysis of RNA segments of human rotaviruses cultivated in cell culture. *J. Gen. Virol.* 60:171.
29. Murabayashi, N., M. Kurita-Taniguchi, M. Ayata, M. Matsumoto, H. Ogura, and T. Seya. 2002. Susceptibility of human dendritic cells (DCs) to measles virus (MV) depends on their activation stages in conjunction with the level of CDw150: role of Toll stimulators in DC maturation and MV amplification. *Microbes Infect.* 4:785.
30. Nakamura, N., A. Yamamoto, Y. Wada, and M. Futai. 2000. Syntaxin 7 mediates endocytic trafficking to late endosomes. *J. Biol. Chem.* 275:6523.
31. Dzionek, A., A. Fuchs, P. Schmidt, S. Cremer, M. Zysk, S. Miltenyi, D. W. Buck, and J. Schumitz. 2000. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. *J. Immunol.* 165:6037.
32. Grabbe, S., E. Kampgen, and G. Schuler. 2000. Dendritic cells: multi-lineal and multi-functional. *Immunol. Today* 21:431.
33. Kadowaki, N., S. Antonenko, and Y. J. Liu. 2001. Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c⁻ type 2 dendritic cell precursors and CD11c⁺ dendritic cells to produce type I IFN. *J. Immunol.* 166:2291.
34. Yi, A.-K., R. Tuetken, T. Redford, M. Waldschmidt, J. Kirsch, and A. M. Krieg. 1998. CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. *J. Immunol.* 160:4755.
35. Pan, B. T., K. Teng, C. Wu, M. Adam, and R. M. Johnstone. 1985. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J. Cell Biol.* 101:942.
36. Sachse, M., G. Ramm, G. Strous, and J. Klumperman. 2002. Endosomes: multipurpose designs for integrating housekeeping and specialized tasks. *Histochem. Cell Biol.* 117:91.
37. Katzmann, D. J., G. Odorizzi, and S. D. Emr. 2002. Receptor downregulation and multivesicular-body sorting. *Nature Rev. Mol. Cell Biol.* 12:893.
38. Escola, J. M., F. Deleuil, E. Stang, J. Boretto, P. Chavrier, and J. P. Gorvel. 1996. Characterization of a lysozyme-major histocompatibility complex class II molecule-loading compartment as a specialized recycling endosome in murine B lymphocytes. *J. Biol. Chem.* 271:27360.
39. van Lith, M., M. van Ham, A. Griekspoor, E. Tjin, D. Verwoerd, J. Calafat, H. Janssen, E. Reits, L. Pastoors, and J. Neeffjes. 2001. Regulation of MHC class II antigen presentation by sorting of recycling HLA-DM/DO and class II within the multivesicular body. *J. Immunol.* 167:884.
40. Kleijmeer, M., G. Ramm, D. Schuurhuis, J. Griffith, M. Rescigno, P. Ricciardi-Castagnoli, A. Y. Rudensky, F. Ossendorp, C. J. M. Melief, W. Stoorvogel, and H. J. Geuze. 2001. Reorganization of multivesicular bodies regulates MHC class II antigen presentation by dendritic cells. *J. Cell Biol.* 155:53.
41. Rehli, M. 2002. Of mice and men: species variations of Toll-like receptor expression. *Trends Immunol.* 23:375.
42. Miettinen, M., T. Sareneva, I. Julkunen, and S. Matikainen. 2001. IFNs activate Toll-like receptor gene expression in viral infections. *Genes Immunity* 2:349.
43. Doyle, S. E., S. A. Vaidya, R. O'Connell, H. Dadgostar, P. W. Dempsey, T.-T. Wu, G. Rao, R. Sun, M. E. Haberland, R. L. Modlin, G. Cheng. 2002. IRF mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* 17:251.
44. Der, S. D., and A. S. Lau. 1995. Involvement of the double-stranded-RNA-dependent kinase PKR in interferon expression and interferon-mediated antiviral activity. *Proc. Natl. Acad. Sci. USA* 92:8841.
45. Yang, Y.-L., L. F. L. Reis, J. Pavlovic, A. Aguzzi, R. Schafer, A. Kumar, B. R. G. Williams, M. Aguet, and C. Weissmann. 1995. Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J.* 14:6095.
46. Sato, M., H. Suemori, N. Hata, M. Asagiri, K. Ogasawara, K. Nakao, T. Nakaya, M. Katsuki, S. Noguchi, N. Tanaka, and T. Taniguchi. 2000. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN- α/β gene induction. *Immunity* 13:539.