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Roles of the Cleaved N-Terminal TLR3 Fragment and Cell Surface TLR3 in Double-Stranded RNA Sensing

Yusuke Murakami,* Ryutaro Fukui,* Yuji Motoi,* Atsuo Kanno,*† Takuma Shibata,* Natsuko Tanimura,* Shin-ichiroh Saitoh,* and Kensuke Miyake*†

TLR3 senses viral dsRNA in endolysosomes. The TLR3 ectodomain is cleaved by proteases such as cathepsins in endolysosomes. It remains controversial whether the N-terminal fragment of TLR3 ectodomain (TLR3N) is cleaved off or remains associated with the C-terminal TLR3 fragment (TLR3C). In addition to endosomes, TLR3 is reported to be expressed on the surface of human fibroblasts, but not of human monocyte-derived dendritic cells. Less is known about roles of TLR3N and cell surface TLR3 in dsRNA sensing. In this study, we show the cleavage site of the TLR3 ectodomain and cell surface expression of TLR3 on mouse primary immune cells. TLR3C, which started at 343S, was associated with TLR3N. Both TLR3N and TLR3C were required for activation of IFN-β and NF-κB promoters by dsRNA, demonstrating that dsRNA is sensed by the TLR3N+C complex. Newly established mAbs to mouse TLR3 revealed that cell surface TLR3 was highly expressed on splenic CD8+ dendritic cells and marginal zone B cells. Cell surface expression of TLR3 on these cells was dependent on the TLR-specific transporter Unc93B1. Although cell surface TLR3 was only weakly expressed on macrophages, TLR3 mAb specifically enhanced TLR3 responses to dsRNA. These results demonstrate that dsRNA is sensed by the TLR3N+C complex and that cell surface TLR3 is a promising target for modulating TLR3 responses.


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Abbreviations used in this article: BM, bone marrow; BM-cDC, BM-derived conventional DC; BM-MC, BM-derived macrophage; CBB, Coomassie Brilliant Blue; DC, dendritic cell; ER, endoplasmic reticulum; HA, hemagglutinin; LRM, leucine-rich motif; LRR, leucine-rich repeat; MZ, marginal zone; NA, nucleic acid; pA, polycytoidal A; PVDF, polyvinylidene difluoride; StAIV, streptavidin.

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expression of TLR3 (21). Despite this progress, cell surface expressions of mouse TLR3 on primary immune cells remain unclarified. Furthermore, functional roles of cell surface TLR3 in responses to dsRNA have not been clear yet.

TLR3 is most highly expressed in CD8+ cross-presenting DCs (22). TLR3 induces rapid and massive production of type 1 IFN and proinflammatory cytokines. TLR3-deficient human patients show that TLR3 is crucial for the defense against HSV-1 encephalitis (23). TLR3 also has a role in sensing UV radiation-dependent cell damage (24). These results demonstrate an essential role of TLR3 in activating immune responses during virus infection and tissue damage. It is important to understand how TLR3 responses are controlled. To study mechanisms regulating TLR3 responses, the current study determined the cleavage site of the TLR3 ectodomain and revealed requirements for TLR3N in dsRNA sensing by TLR3. Furthermore, cell surface TLR3 on CD8+ DCs and marginal zone (MZ) B cells was detected by novel mAbs against mouse TLR3. TLR3 mAb enhanced dsRNA-dependent TLR3 responses without any activation with TLR3 mAb alone, suggesting that cell surface TLR3 can be a target for modulating TLR3 responses.

Materials and Methods

Generation of anti-mouse TLR3 mAbs

To establish anti-mouse TLR3 mAbs, BALB/c background Tlr3-/- mice were immunized with Ba/F3 cells expressing Flag-6His-conjugated mouse TLR3 (Ba/F3-mTLR3H) in CFA/IFA used as adjuvants. Next, to boost the immunization, the mice were immunized three times with Ba/F3-mTLR3H in PBS. Three days after the final immunization, splenic cells were fused with SP2/O myeloma cells. Hybridomas producing anti-TLR3 mAb were selected by flow cytometry staining of Ba/F3 cells expressing mTLR3fH. Three days after the final immunization, the mice were immunized three times with Ba/F3-mTLR3fH in CFA/IFA used as adjuvants. Next, to boost the immunization, the mice were immunized three times with Ba/F3-mTLR3H in PBS. Three days after the final immunization, splenic cells were fused with SP2/O myeloma cells. Hybridomas producing anti-TLR3 mAb were selected by flow cytometry staining of Ba/F3 cells expressing mTLR3fH. The novel mAbs, named CaT3 and PaT3, were used in this study.

Mice, reagents, and cells

C57BL/6N mice were purchased from Japan SLC, and Tlr3-/- mice on C57BL/6N background were provided by S. Akira (Osaka University, Osaka, Japan). The Unc93b1-/- mice were provided by B. Beutler (University of Texas, Dallas, TX) and the Scripps Research Institute. All animal experiments were conducted with the approval of the Animal Research Committee of the Institute of Medical Science, University of Tokyo.

Biotinylated anti-Flag Ab (clone M2) and anti-Flag M2 agarose affinity gel were purchased from Sigma-Aldrich. Rabbit GFP polyclonal Ab (pAb) and mAb (clone FM2/04) were purchased from Invitrogen and MBL, respectively. The pAb to the N-terminal fragment of TLR3 (TLR3N) was purchased from Millipore.

J774 cells were cultured in high-glucose DMEM supplemented with 10% FBS, penicillin-streptomycin-glutamine (Life Technologies), and 50 μM 2-ME. Ba/F3 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin-streptomycin-glutamine, and 50 μM 2-ME. Bone marrow (BM)-derived macrophage (BM-MC) was prepared. In brief, BM cells were plated at 1 × 10^6 cells per 10 ml with 10% FCS-DMEM supplemented with 100 ng/ml mouse rM-CSF (PeproTech, Rocky Hill, NJ) in 10-cm cell culture dishes.

Plasmid constructs

The C terminus of mouse TLR3, 7, 8, and 9 was tagged with Flag-6His. They were amplified by PCR and cloned into retroviral pMX, pMXpuRO, or pMXneo vectors (provided by Dr. T. Kitamura, Tokyo, Japan). We also constructed the short hairpin RNA-targeting Unc93b1 in retroviral vector pSSEH. For the constructs encoding TLR3 N fragments (Figs. 3 and 4), TLR3 fragments corresponding to aa 1–342 (TLR3N342), 1–346 (TLR3N346), or 1–356 (TLR3N356) were cloned into pMX vector. For the TLR3C constructs, TLR3 fragments corresponding to aa 1–35, the signal peptide, and following 10 aa, fused to aa 343–905 (TLR3C343), 347–905 (TLR3C347), or 357–905 (TLR3C357), were cloned into the pMX vector using HA-tag. The In-Fusion HD cloning kit (Clontech Laboratories) and Rapid DNA Ligation kit (Roche Applied Science) were used for cloning.

Retroviral transduction

pMX and pMXpuRO vectors were transfected into Plat-E packaging cells with FuGene6 (Roche Applied Science). After 2 d of incubation, supernatants were obtained as virus suspensions. Ba/F3 and BaB cells were infected by virus suspensions mixed with DOTAP (Roche Applied Science).

N-terminal amino acid sequencing

TLR3-GFP was stably expressed in the RAW264.7 cell line. Next, 5 × 10^5 transfectants were collected and lysed in the buffer, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1.0% Triton-X-100, and 1× complete EDTA-free mixture (Roche Applied Science). Anti-GFP agarose beads were added to the lysate and incubated overnight at 4˚C. Beads were collected and washed twice by washing buffer, 150 mM NaCl, 30 mM Tris-HCl (pH 7.4), and 0.1% Triton X-100, and bound protein was eluted in elution buffer, 150 mM NaCl, 30 mM glycine-HCl (pH 2.5), and 0.1% Triton X-100 (1% 1 M Tris-HCl (pH 8.0)). Eluted sample was concentrated and dialyzed in buffer (10 mM NaCl, 0.1% Triton-X-100) by Amicon Ultra (Millipore). Concentrated sample was dissolved in the sample buffer, 2% SDS, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8), 0.025% bromophenol blue, and 5% 2-ME, and subjected to SDS-PAGE. Separated protein was transferred to polyvinylidene difluoride (PVDF) membranes. Transferred protein was visualized by Coomassie brilliant blue, and the membrane containing TLR3N and 3C was cut out. The membrane was subjected to N-terminal amino acid sequencing by Edman degradation (APRO Science).

Immunoprecipitation

Cells were collected and washed with 1× PBS. Washed cells were lysed with 1% Triton X-100 lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 10% glycerol, 1 mM DTT, and 1× complete EDTA-free mixture (Roche Applied Science)]. After incubation for 30 min on ice, lysates were centrifuged and debris was removed. The cell lysates were rotated for 2 h at 4 °C with beads coupled with anti-Flag or anti-TLR3 mAbs. The beads were then washed with 0.1% Triton X-100 washing buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 10% glycerol, 1 mM DTT] three times. Immunoprecipitates were subjected to SDS-PAGE after boiling in reduced sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% 2-ME, and 0.005% bromophenol blue] or nonreduced sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, and 0.005% bromophenol blue]. After electrophoresis, samples were transferred to PVDF membranes and subjected to immunoblotting.

Luciferase assay

HEK293 cells (1 × 10^6 cells/well) were transiently transfected in 6-well plates using Lipofectamine 2000 reagent (Invitrogen) with pMX, pMX-TLR3, or -TLR3 mutant vector (5 μg), together with a luciferase-linked IFN-β promoter reporter gene, p551B-Luc (100 ng). Twenty-four hours after transfection, cells were harvested and seeded into 96-well plates (5 × 10^3 per well). After 24 h, cells were stimulated with medium alone or poly(I:C) for 6 h. Stimulated cells were lysed using lysis buffer (Promega), and luciferase activities were measured. Data are expressed as mean values with SD.

ELISA

J774 cells and BM-MCs plated at 5 × 10^5 per well on 96-well plates were stimulated with indicated TLR ligands for 24 h. The supernatant was subjected to ELISA for RANTES production (R&D Systems).

Cell staining and flow cytometry

BM cells and splenocytes were stained with fluorescein-conjugated Abs specific for the following markers in flow cytometry analysis: CD4 (GK1.5), CD8a (53-6.7), CD11b (M1/70), CD11c (HL3), PDCA-1 (927), B220 (RA3-68B), CD21 (7G6), CD23 (B3B4), Ly6C (HK1.4), Ly6G (1A8), and Gr-1 (RB6-8C5). Biotinylated anti-TLR3 (CaT3 and PaT3) mAbs were established in this study. For preparation of single-cell splenocyte suspensions, spleens were minced by scissors and incubated for 30 min at 37°C in RPMI 1640 with 0.09 U/ml –1 Liberase TL (Roche) and 0.1 mg/ml –1 DNase I (Roche). Finally, suspended splenocytes were teased through nylon mesh after pipetting and subjected to cell staining.

The cell staining was performed in staining buffer, 1× PBS with 2.5% FBS and 0.1% NaN3. Single-cell suspensions were incubated on ice for 15 min with biotinylated mAbs diluted in staining buffer. Then cells were washed and incubated with PE-streptavidin (BD Biosciences) and fluorescein-conjugated mAbs for 15 min. Stained cells were analyzed by the FACSCalibur or FACSaria flow cytometers (BD Biosciences).

Cell surface biotinylation and precipitation with StAve

Wild-type, Tlr3-/-, or Unc93b1-/-/M mice were washed twice with HBSS and subjected to cell surface biotinylation with EZ-Link Sulfo-
NHS-LC-LC Biotin (Thermo Scientific Protein Biology Products) for 1 h at 4°C. After washing with HBSS, BM-MCs were lysed in 1% Triton X-100 lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10% glycerol, 1 mM DTT; and 1× Complete EDTA-free mixture [Roche Applied Science]]. Cell lysate was incubated on ice for 30 min and centrifuged to remove debris. Supernatant was collected and rotated at 4°C for 2 h with StA Alumni conjugated Dynabeads (Veritas). After incubation, beads were washed three times with 0.1% Triton X-100 washing buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10% glycerol, and 1 mM DTT). Immunoprecipitates were subjected to SDS-PAGE after boiling in sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 4% SDS, and 0.005% bromophenol blue). After electrophoresis, samples were transferred to PVDF membrane and subjected to immunoblotting with TLR3N pAb.

**Immobilized Abs**

PaT3 or CaT3 was coated at 10 µg/ml on the 96-well plate and incubated at 37°C for 1 h. Then the plates were washed twice with 1× PBS.

**Quenching of Alexa488-labeled mAbs**

J774 cells were cultured with Alexa488-labeled Abs at 4°C or 37°C for 24 h. Then these cells were washed twice with 1× PBS and stained with Anti-Alexa488 Ab (Life Technologies) at 4°C for 20 min. After washing the cells, mAb uptake was measured by the FACSCalibur flow cytometers (BD Biosciences).

**Immunoprecipitation of TLR3 mAb-binding TLR3**

J774 cells were cultured with CaT3, PaT3, or IgG1 control Ab at 37°C for 24 h. After washing with 1× PBS, these cells were lysed in 1% Triton X-100 lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10% glycerol, 1 mM DTT; and 1× Complete EDTA-free mixture (Roche Applied Science)]. Cell lysate was incubated on ice for 30 min and centrifuged to remove debris. Supernatant was collected and rotated at 4°C for 8 h with protein G Sepharose 4 Fast Flow (Amersham Biosciences). After incubation, beads were washed three times with 0.1% Triton X-100 washing buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10% glycerol, and 1 mM DTT). Immunoprecipitates were subjected to SDS-PAGE after boiling in sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 4% SDS, and 0.005% bromophenol blue). After electrophoresis, samples were transferred to PVDF membrane and subjected to immunoblotting with TLR3N pAb.

**Statistical analysis**

Data from triplicate samples were statistically analyzed by Student t test. The p value <0.01 was considered to be significant.

**Results**

**Establishment of novel anti-mouse TLR3 mAbs**

To study endogenous TLR3, novel two mAbs against mouse TLR3 were established. The specificity of the mAbs was confirmed by membrane-permeabilized staining (Fig. 1A). The mAbs, CaT3 and PaT3, reacted to TLR3, but not TLR7, TLR8, or TLR9. Neither PaT3 nor CaT3 showed cross-reactivity with human TLR3. Furthermore, PaT3 specifically binds to TLR3N+C, demonstrating that this signal is TLR3N. These results demonstrate that CaT3 and PaT3 are able to bind to both uncleaved and cleaved TLR3.

**Determination of the cleavage site of the TLR3 ectodomain**

To study TLR3 proteolytic cleavage, the N-terminal sequence of the cleaved TLR3 fragments was determined. TLR3-GFP was expressed in RAW264.7 macrophage cell line and purified with anti-GFP mAb. Eluted fractions were analyzed by SDS-PAGE and Coomassie Brilliant Blue (CBB) staining (Fig. 2A). Immunopробing with anti-GFP Ab identified TLR3F-GFP and TLR3C-GFP. The ~70-kDa signal was predicted as TLR3N from its apparent molecular mass. The N-terminal amino acid sequences of TLR3C and TLR3N were determined. TLR3C and TLR3N started at 343S and 26T, respectively (Fig. 2B). The TLR3 ectodomain consists of tandem repeats of 22 leucine-rich repeats (LRRs). TLR3C contains LRR12-LRR22 (Fig. 2C). The result that TLR3N started at 26T experimentally demonstrated the signal peptide cleavage site at 25S.

**PaT3 specifically binds to TLR3N**

Based on the determined amino acid sequence, TLR3N or TLR3C was expressed in Ba/F3 cells and subjected to membrane-permeabilized staining. PaT3 bound to TLR3N, but not TLR3C (Fig. 2D), whereas CaT3 bound to neither fragment. CaT3, however, reacted with the Ba/F3 cells coexpressing TLR3N and TLR3C (Fig. 2D). PaT3 and CaT3 did not interfere with the binding of the other mAb, demonstrating that these two mAbs react with completely different epitopes on the TLR3 ectodomain (Supplemental Fig. 2).

**Independently expressed TLR3N and TLR3C were associated with each other**

According to the previous reports (14, 15), dsRNA sensing by TLR3C alone is still controversial. One report shows that TLR3C responds to dsRNA (15), whereas another report shows that TLR3C fails to respond to dsRNA (14). After TLR3 cleavage, TLR3N is suggested to be still associated with TLR3C (14), but the requirement of TLR3N for TLR3 responses has not been clarified. We previously showed that the TLR9N+C complex, but not TLR9C alone, responds to ssDNA (10). Expression of TLR9N enables TLR9 responses in TLR9C-expressing cell lines. Furthermore, TLR9 responses in TLR9−/− BM-cDCs are complemented by TLR9N and TLR9C, but not by TLR9C alone. These previous results indicate that coexpression of TLR3N and TLR3C leads to the assembly of TLR3N+TLR3C complex. Notably, CaT3 reacted with the cells expressing both TLR3N and TLR3C, but not those expressing TLR3N or TLR3C alone (Fig. 2D). These results suggest that CaT3 binds to TLR3N+C complex. To confirm that separately expressed TLR3N and TLR3C are associated with each other, TLR3N and TLR3C-hemagglutinin (HA) were expressed in Ba/F3 cells and immunoprecipitated by PaT3 or anti-HA, and coprecipitation of the other TLR3 fragment was detected. TLR3N alone showed faster migration than TLR3N endogenously cleaved from TLR3 (Fig. 2E, lanes 4 and 5), probably due to distinct posttranslational modification such as glycosylation. TLR3C was coprecipitated with TLR3N by PaT3 (Fig. 2E, lane 4), whereas coprecipitation of TLR3N with TLR3C was difficult to detect (Fig. 2E, lane 9). PaT3 is likely to stabilize the association between TLR3N and TLR3C (see below).

**Requirement of TLR3N for dsRNA responses**

To address a role of TLR3N in dsRNA sensing by TLR3C alone, we studied the luciferase reporter plasmid p55C1B for IFN-β promoter activity. Comparable expression of TLR3 or its fragments was evaluated by membrane-permeabilized staining with PaT3 or anti-HA mAb (Supplemental Fig. 3). dsRNA-dependent IFN-β promoter activation was not found in HEK293 cells expressing TLR3N truncated at 342, 346, or 356 (N342, N346, or N356) or those expressing TLR3C starting at 343, 347, or 357 (C343, C347, or C357). TLR3 responses were observed by coexpression of N342 with C343, N346 with C347, or N356 with C343 (Fig. 3A).
TLR3-dependent NF-κB activation was next studied by using Ba/F3 cells expressing the reporter construct NF-κB-GFP. TLR3, TLR3N, and TLR3C were expressed in this cell line, and expression of each fragment was confirmed by membrane-permeabilized staining with Pat3 for TLR3N and anti-HA for TLR3C (Supplemental Fig. 4). These cells were stimulated with poly(I:C), and GFP induction was evaluated by flow cytometry (Fig. 3B). Poly(I:C)-dependent GFP induction was not seen in cells expressing any single fragment. TLR3-dependent GFP induction was detected by combined expression of N342+C343 or N346+C347, but not of N356+C357 (Fig. 3B). These results are consistent with those obtained by IFN-β induction assay and demonstrate that dsRNA is sensed by the TLR3N+C complex, not by TLR3C alone.

**Cell surface expression of TLR3 on CD8+ DCs and MZ B cells**

Human TLR3 is expressed on the surface of fibroblasts, but not of DCs, and TLR3 mAb inhibits TLR3 responses in fibroblasts (19, 20), suggesting a functional role of cell surface TLR3. In contrast, cell surface expression of mouse TLR3 on primary immune cells has never been reported. In a previous study, membrane-permeabilized staining with anti-TLR3 shows that CD8+ cDC subset expresses TLR3 more highly than other cDC subsets (22). We performed cell surface and membrane-permeabilized staining of splenocytes with PaT3. Membrane-permeabilized staining revealed that TLR3 was expressed in cDCs, but not pDCs (Fig. 4A). The specificity of staining was verified by staining Tlr3−/− DCs. Consistent with the previous report (22), highest TLR3 expression was observed in CD8+ cDCs. Cell surface TLR3 was also detected on BM-MCs (Fig. 4C). Although TLR3 was detectable...
by membrane-permeabilized staining of BM-derived cDCs (BM-cDCs), cell surface TLR3 was hard to detect.

Unc93B1 is required for cell surface expression of cleaved TLR3

To gain insight into a mechanism regulating cell surface expression of TLR3, Unc93b1<sup>345/345</sup> mice were studied by using PaT3. In Unc93b1<sup>345/345</sup> mice harboring loss-of-function mutation, NA-sensing TLRs fail to respond to NAs, because all the NA-sensing TLRs failed to exit the ER (16, 17). Cell surface TLR3 was not detected on splenic CD8<sup>a</sup> + cDCs (Fig. 4D), demonstrating an essential role of Unc93B1 in cell surface expression of TLR3.

Next, we asked the origin of cell surface TLR3. If cell surface TLR3 comes from the ER, uncleaved TLR3 is likely to be enriched on the cell surface. BM-MCs were subjected to cell surface biotinylation, immunoprecipitation with StAv or PaT3, and immunoprecipitation with anti-HA mAb and immunoprecipitation with anti-HA (top) or anti-TLR3 (bottom) Abs.

**FIGURE 2.** TLR3C starts at 343S, and Pat3 mAb binds to TLR3N. (A) TLR3-GFP expressed in the RAW264 macrophage line was subjected to purification by anti-GFP mAb. Eluted fractions (Fr. 1–5) were analyzed by SDS-PAGE and CBB staining. The right panel shows immunoprobng with GFP mAb of the immunoprecipitated with GFP mAb from RAW cells expressing TLR3-GFP (lane 1) or plain RAW264 (lane 2). (B) The amino acid sequences of mouse and human TLR3. The determined N-terminal amino acid sequences of TLR3C and TLR3N are underlined. The signal peptide is indicated by a line. (C) Schematic representation of proteolytically cleaved TLR3. (D) Open histograms show membrane-permeabilized staining with anti-TLR3 mAbs or anti-HA mAb of Ba/F3 cells expressing TLR3, TLR3N, or TLR3C-HA. TLR3C-HA lacks the region from 36 to 342. Gray histograms show staining with the second reagent alone. (E) Ba/F3 cells expressing TLR3N, TLR3C-HA, or TLR3F-HA were subjected to immunoprecipitation with anti-TLR3N or anti-HA mAb and immunoprecipitation with anti-HA (top) or anti-TLR3 (bottom) Abs.
blotting with anti-TLR3 Ab. In Unc93b1<sup>3d/3d</sup> BM-MCs, TLR3 was not detected in the precipitate with StAv (Fig. 4E, lane 2), demonstrating cell surface–specific biotinylation. Only cleaved TLR3 was detected by immunoprecipitation with StAv, and no increase in uncleaved TLR3 was observed when compared with immunoprecipitation with PaT3 (Fig. 4E, compare upper and middle lane 1), indicating that cleaved TLR3 is a predominant form on the cell surface. These results demonstrate that cleaved TLR3 is predominantly expressed on the cell surface.

**PaT3 enhances TLR3 responses**

The importance of cell surface TLR3 in dsRNA sensing was addressed by studying effects of PaT3 and CaT3 on TLR3 responses. Cell surface TLR3 was detected on J774 cells. J774 cells were stimulated with poly(I:C) in the presence of TLR3 mAb, and upregulation of costimulatory molecules and RANTES production were analyzed. Interestingly, PaT3, but not CaT3, augmented poly(I:C)-dependent upregulation of CD40 and CD69 (Fig. 5A). RANTES production in response to poly(I:C) was also upregulated by PaT3 (Fig. 5B). RANTES production induced by lipid A was not altered by TLR3 mAbs. Next, the effect of TLR3 mAb on poly(I:C) responses of BM-MCs was studied. Although cell surface TLR3 was only weakly detected on BM-MCs (Fig. 4C), poly(I:C)-induced RANTES production was augmented by PaT3 (Fig. 5C). PaT3 did not enhance lipid A–dependent RANTES production by BM-MCs. Augmentation of IFN-β mRNA induction by

**FIGURE 3.** dsRNA sensing by TLR3N+C. (A) HEK293 cells were transfected with the reporter plasmid for IFN-β promoter and indicated expression vectors. The TLR3 fragments used were TLR3N truncated at 342, 346, or 356 (N342, N346, and N356) and TLR3C starting at 343, 347, or 357 (C343, C347, and C357). Transfected cells were stimulated with poly(I:C), as indicated. Luciferase activity was determined. The results are represented by average value with SD. (B) Ba/F3 cells expressing indicated TLR3 fragments and NF-κB-GFP were stimulated with poly(I:C) or a TLR2 ligand PamCSK4. NF-κB–dependent GFP induction was determined by flow cytometry.
Pat3 was also observed (Fig. 5C). In contrast to these strong effects, Pat3 alone did not induce any activation in J774 and BM-MCs.

To gain insight into a mechanism underlying the Pat3-dependent enhancement of TLR3 responses, Pat3 internalization was first studied. J774 cells were incubated with Alexa488-labeled Pat3 or Cat3 at 4 or 37˚C and analyzed by flow cytometry after quenching the fluorescence of the mAb on the cell surface. All the mAbs, including control IgG1 mAb, were internalized at 37˚C (Fig. 6A, lower). Considering that quenching did not alter fluorescence intensity, the mAb were unlikely to remain on the cell surface. To confirm that internalized anti-TLR3 mAbs still bind to TLR3, internalized mAbs were precipitated by protein G and immunoblotting with anti-TLR3 Ab was performed. TLR3 was precipitated by Cat3 or Pat3, but not by IgG1 control mAb (Fig. 6B). These results suggest that internalized anti-TLR3 mAbs still bind to TLR3.

Requirement of Pat3 internalization in the enhancing effect of Pat3 was next asked. J774 cells were stimulated with immobilized Pat3. Immobilized Pat3 would ligate TLR3 on the cell surface without internalization. No amplification was observed by immobilized Pat3 (Fig. 6C), suggesting that Pat3 internalization is required for the enhancing effect of Pat3.

Considering that Pat3, but not Cat3, showed the enhancing effect, it is important to find the Pat3-specific effect on TLR3. TLR3N was required for TLR3 responses, and association between TLR3N and TLR3C was more clearly demonstrated by Pat3 than anti-HA Ab capturing TLR3C (Fig. 2E). We hypothesized that Pat3 may strengthen the association between TLR3N and TLR3C. To address this possibility, TLR3C-HA was immunoprecipitated with anti-HA Ab in the presence of Pat3 or Cat3 mAb from the Ba/F3 cells expressing TLR3C-HA and TLR3N. Coprecipitation of TLR3N with TLR3C was clearly enhanced in the presence of Pat3, but not Cat3 (Fig. 6D). Poly(I:C) stimulation did not alter TLR3N coprecipitation. These results indicate that Pat3 enhances TLR3 signaling in endosomes by stabilizing the association between TLR3N and TLR3C, indicating an im-

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**FIGURE 4.** Cell surface expression of TLR3. (A–C) Splenic cells from wild-type or Tlr3<sup>−/−</sup> mice were subjected to cell surface and membrane-permeabilized staining with anti-TLR3 mAb Pat3 together with cell type markers. Open histograms show TLR3 expression on DC subsets (A), B cell subsets (B), BM-MCs (C), and BM-cDCs (C). (D) Open histograms show cell surface TLR3 on splenic CD8<sup>+</sup> cDCs from indicated mice. (E) BM-MCs from wild-type, Unc93b1<sup>−/−</sup>, or Tlr3<sup>−/−</sup> mice were subjected to cell surface biotinylation, immunoprecipitation with StAv (upper) or Pat3 (middle), and immunoblotting with anti-TLR3 pAb (upper and middle). Whole-cell lysates are immunoprobred with anti-EEA1 pAb as a loading control.
portant role of association between TLR3N and TLR3C in TLR3 responses.

Discussion

TLR ectodomains consist of LRRs, the tandem repeats of a leucine-rich motif (LRM). LRM is typically 24-aa length and adopts a loop structure, beginning with a short β-strand followed by a β-turn (25). The extra residues in LRM form a loop that protrudes from the LRR solenoid. A tentative loop in TLR9 ectodomain is located between LRR14 and LRR15 and shown to be cleaved (10). A similar loop is located in the LRR11 of the TLR3 ectodomain, which is predicted to be cleaved (14). Consistent with this prediction, our present study experimentally determined the cleavage site at 343S, which resides in the protruding loop of LRR11.

Association of TLR3N and TLR3C was previously suggested by the result that cleaved TLR3 fragments are detectable only under denatured, but not nondenatured condition (14). However, proteolytic cleavage of the TLR3 ectodomain in endolysosomes leads to the presence of all the fragments, including uncleaved TLR3 (TLR3F), TLR3N, and TLR3C. It is difficult to exclude a possibility that TLR3N and TLR3C are indirectly associated with each other by forming a complex with uncleaved TLR3F. In the current study, the determination of the cleavage site enabled separate expression of TLR3N and TLR3C. Thereby, the association of TLR3N and TLR3C was directly shown. Interestingly, coprecipitation of TLR3N was hard to detect in immunoprecipitation of TLR3C by the Ab to C-terminal–tagged HA. Interaction between TLR3N and TLR3C may be unstable in the cell lysate. PaT3 was able to enhance TLR3N coprecipitation with TLR3C, indicating that PaT3 stabilizes the interaction between TLR3N and TLR3C. The persistent association of TLR3C and TLR3N was further supported by another TLR3 mAb Cat3, which is likely to react with the TLR3N+C complex, but not with either fragment. In the TLR ectodomains, tandem repeats of LRM form a solenoid structure with parallel β-sheets. TLR ectodomains are stabilized by their interior hydrophobic cores and hydrogen bonds between parallel

![FIGURE 5. TLR3 mAb enhances poly(I:C) responses.](http://www.jimmunol.org/)

(A and B) J774 cells were stimulated by poly(I:C) or lipid A at the indicated concentrations together with PaT3 (△), CaT3 (▴), and control IgG1 (X), or without Ab (♦) for 24 h. Cell surface CD40 and CD69 were stained. The results are presented by MFI value. RANTES production was measured by ELISA. The results were presented by the average values with SD from triplicate wells. (C) BM-MCs were stimulated with poly(I:C) or lipid A together with PaT3 (△), CaT3 (▴), and control IgG1 (X), or without Ab (♦) for 24 h in ELISA or indicated times in quantitative PCR. RANTES production was measured by ELISA. (D) For IFN-β mRNA induction, mRNA was extracted at 0, 3, and 6 h after poly(I:C) stimulation. IFN-β mRNA was quantitated by quantitative PCR, and the values are normalized by hypoxanthine phosphoribosyltransferase mRNA.
TLR3 needs to be within the protruding loop, not in the LRRs portion of TLR3C. These results suggest that the cleavage site of TLR3N association with TLR3C by competing with the N-terminal paired TLR3 responses. The extended portion may also impair the LRR structure. Similarly, the 14-aa extension of TLR3N im-TLR3C locates in the LRRs, not the protruding loop, 14-aa de-TLR3N+C responses. Considering that the N-terminal portion of promoters. Just 14-aa N-terminal deletion in TLR3C abolished and subjected to immunoprecipitation with TLR3 mAb PaT3, CaT3, or no Abs for 2 h at 4˚C treated with PaT3, CaT3, or IgG1 control mAb for 24 h. Internalized mAbs were precipitated by protein G, and immunoblotting with anti-TLR3 Ab was performed. Actin is shown as a loading control. (C) J774 cells were stimulated with poly(I:C) at the indicated concentrations without Ab (●) or together with soluble PaT3 (▲), immobilized PaT3 (●), or immobilized CaT3 (■) for 24 h. RANTES production was measured by ELISA. The results were represented by the average values with SD from triplicate wells. (D) BaF3 cells expressing TLR3N and TLR3C were stimulated with or without poly(I:C) at 10 μg/ml for 1 h. Cell lysates were treated with PaT3, CaT3, or no Abs for 2 h at 4˚C and subjected to immunoprecipitation with anti-HA mAb, followed by immunoblotting with anti-TLR3 pAb (top) or anti-HA mAb (bottom).

Immunoprecipitation of endogenous TLR3 shows that a majority of TLR3 is cleaved in BM-MCs, probably due to rapid degradation of uncleaved TLR3 (13). Consistent with this, cell surface biotinylation shows that cell surface TLR3 is mostly cleaved on these cells. Previous study also shows cleavage of cell surface TLR3 (13). These results indicate that TLR3 traffics from endolysosomes to the cell surface. Considering that PaT3 mAb enhanced TLR3 responses, cell surface TLR3 is likely to be activated by dsRNA probably after internalization, suggesting the shuttling of the TLR3N+C complex between the endolysosomal systems and the plasma membrane. Cell surface TLR3 may be a target for enhancing or inhibiting TLR3 responses. Given that TLR3 promotes cross-priming in viral infection (26), targeting cell surface TLR3 by TLR3 mAb may deserve further study in terms of vaccine development.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Knock-down efficiency of Unc93B1 in J774 cells
J774 cells were knocked down by shRNA. Unc93B1 knockdown efficiency was confirmed by quantitative PCR.
Supplementary Figure 2. The epitopes of PaT3 and CaT3 are distinct from each other. mTLR3 overexpressed Ba/F3 cells were subjected to membrane-permeabilized staining with biotinylated CaT3 or PaT3. Before addition of biotinylated mAb, cells were pretreated with 100 μg CaT3 or PaT3 as indicated. Gray histograms show staining with the second reagent alone.
Supplementary Figure 3. Expression of TLR3 fragments in HEK293 cells
HEK293 cells were transfected with indicated expression vectors. Expression of
TLR3 fragments were studied by membrane permeabilized staining with PeT3 for
TLR3N and anti-HA for TLR3C. Gray histograms show staining with the second
reagent alone.

Figure S3. Murakami et al.
Supplementary Figure 4. Expression of TLR3 fragments in Ba/F3 cells
Ba/F3 cells were transfected with indicated expression vectors. Expression of TLR3 fragments were studied by membrane permeabilized staining with PaT3 for TLR3N and anti-HA Ab for TLR3C. Gray histograms show staining with the second reagent alone.