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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. The Journal of Immunology, 2014, 193: 5208–5217.

To compartmentalize NA sensing by TLR3s in endolysosomes, two mechanisms have been proposed. The first mechanism depends on the proteolytic cleavage of TLRs in endolysosomes (6, 7). NA sensing is activated by proteolytic cleavage of TLR3 ectodomains after trafficking to endolysosomes. After proteolytic cleavage, requirements of N-terminal TLR fragments in NA sensing have been controversial. For example, the ectodomain of TLR9, a sensor for ssDNA, is cleaved by asparagine endopeptidase and/or cathepsins in endolysosomes (8, 9). Although TLR9C alone is reported to sense DNA in the previous study (7), another study showed that the N-terminal TLR9 fragment (TLR9N) is cleaved, associated with C-terminal TLR9 fragment (TLR9C) and that TLR9N association is essential for DNA sensing (10). TLR7, a ssRNA sensor, is also proteolytically cleaved (11). The TLR7N fragment is linked to the TLR7C fragment by a disulfide bond, which is indispensable for TLR7 proteolytic cleavage and responses (11). Furthermore, the structure of the complex consisting of TLR8 and a small chemical ligand has revealed that TLR8 is also cleaved, but TLR8N is associated with TLR8C (12). TLR8N and TLR8C both have ligand binding domains and dimerization interfaces (12), demonstrating an indispensable role of TLR8N in ligand binding and signaling. TLR3, a dsRNA sensor, is cleaved, but the precise cleavage site has been only suggested (13, 14). TLR3N is suggested to be associated with TLR3C and required for dsRNA sensing (14, 15). However, the direct evidence for TLR3N requirement in dsRNA sensing has not been shown.

The second mechanism for compartmentalization of NA sensing is based on TLR transportation. Trafficking of NA-sensing TLRs out of endoplasmic reticulum (ER) is regulated by Unc93B1, a multiple transmembrane protein (16, 17). In Unc93B1−/− mice harboring loss-of-function mutation, NA-sensing TLRs fail to respond to NAs, because all the NA-sensing TLRs remain uncleaved in the ER (16, 17). Enforced TLR9 localization to the cell surface causes systemic lethal inflammation (18). Nonetheless, cell surface expression of TLR9 on splenic dendritic cells (DCs) is recently shown (10), suggesting a functional role of cell surface TLR9 in ssDNA sensing. Human TLR3 is also shown to be expressed on the surface of fibroblasts, but not of DCs (19, 20). Moreover, human TLR3 overexpressed in HEK293 cells is shown to be expressed on the cell surface (13, 21). Unc93B1 overexpression augments cell surface...
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<sup>+</sup> cross-presenting DCs (22). TLR3 induces rapid and massive production of type I 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<sup>+</sup> 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 Tbr<sup>3<sup>-/−</sup></sup> mice were immunized with Ba/F3 cells expressing Flag-6 His. The C terminus of mouse TLR3, 7, 8, and 9 was tagged with Flag-6 His. Plasmid constructs

![Image](https://example.com/image)

BM cells were cultured in RPMI 1640 medium supplemented with 100 ng/ml mouse rM-CSF (PeproTech, Rocky Hill, NJ) and 10% FBS, penicillin-streptomycin-glutamine, and 50 µM 2-ME. Ba/F3 cells were cultured in RPMI 1640 medium supplemented with SP2/O myeloma cells. Hybridomas producing anti-TLR3 mAb were in PBS. Three days after the final immunization, splenic cells were fused with Ba/F3-mTLR3fH in CFA/IFA used as adjuvants. Next, to boost the response, mice were immunized with Ba/F3 cells expressing Flag-6 His. Hybridomas producing anti-TLR3 mAb were selected by flow cytometry staining of Ba/F3 cells expressing mTLR3H.

**N-terminal amino acid sequencing**

TLR3-GFP was stably expressed in the RAW264.7 cell line. Next, 5×10<sup>4</sup> 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 1% Triton X-100, and bound protein was eluted in elution 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 twice 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 CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 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 CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 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<sup>4</sup> 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, p53CB1-Luc (100 ng). Twenty-four hours after transfection, cells were harvested and seeded into 96-well plates (5×10<sup>3</sup> 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<sup>5</sup> 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), CD8<sub>a</sub> (53-6.7), CD11c (M170), CD11c (HL3), PDCA-1 (927), B220 (RA3-68B2), CD21 (7G6), CD23 (B3B4), Ly6C (HK1.4), Ly6G (1A8), and Gr-1 (RB6-8C5). Biotinylated anti-TLR3 (CaT3 and PaT3) mAbs were purchased from BioLegend. Stained cells were analyzed by FACScalibur or FACSAria flow cytometers (BD Biosciences).

**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 Ba/E cells were infected with virus suspensions mixed with DOTAP (Roche Applied Science).

**Cell surface biotinylation and precipitation with StAv**

Wild-type, TLR3<sup>-/−</sup>, or Unc93b<sup>1<sup>−/−</sup></sup> BM-MCs were washed twice with HBSS and subjected to cell surface biotinylation with EZ-Link Sulfo-
Proteolytic Cleavage and Cell Surface Expression of TLR3

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 StAv-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.

Immmobilized 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 FACS Calibur 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, the mAbs immunoprecipitated TLR3, but not TLR7, from a pro-B cell line Ba/F3 expressing TLR3 or TLR7 (Fig. 1B). Endogenous TLR3 was next precipitated from J774 macrophage cell line by TLR3 mAbs and immunoprecipitated with the pAb against TLR3N. Two signals were detected in J774 cells (Fig. 1C). We also studied Unc93B1-silenced J774, which showed ∼90% reduction in Unc93B1 mRNA (Supplemental Fig. 1). The ∼120-kDa signal was immunoprecipitated from Unc93B1-silenced J774 cells and is likely to be full-length, uncleaved TLR3 (TLR3F) in the ER. The ∼70-kDa signal disappeared in Unc93B1-silenced J774 cells (Fig. 1C), indicating 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 the role of TLR3N in dsRNA sensing by TLR3, TLR3N and TLR3C were separately expressed in HEK293 cells together with the luciferase reporter plasmid pS5C1B 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, but not by coexpression of N356 with C357 or N342 with C357 (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 detected on CD8+ cDCs and very weakly on the other cDC subsets (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 detected on CD8+ cDCs and very weakly on the other cDC subsets (Fig. 4A). Splenic B cell subsets were next studied. TLR3 was detected in both follicular and MZ B cells by membrane-permeabilized staining, whereas only MZ B cells expressed TLR3 on the cell surface (Fig. 4B). 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>343<sup>3d</sup></sup> mice were studied by using PaT3. In Unc93b1<sup>343<sup>3d</sup></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>+</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 immunoprobings.
blotting with anti-TLR3 Ab. In Unc93b13d/3d 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 in 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.](image)

(A) HEK293 cells were transfected with the reporter plasmid for IFN-β promoter and indicated expression vectors. The TLR3 fragments used are 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 Pam3CSK4. 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 mAbs were unlikely to remain on the cell surface. To confirm that internalized anti-TLR3 mAbs still bound to TLR3, internalized mAbs were precipitated by protein G and immunoblotted 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-
important 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.

TABLE 4. TLR3 mAb enhances poly(I:C) responses. (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 represented by MFI value. RANTES production was measured by ELISA. The results were represented 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.

FIGURE 5. TLR3 mAb enhances poly(I:C) responses. (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 represented by MFI value. RANTES production was measured by ELISA. The results were represented 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.
β-sheets of LRRs (25). Proteolytic cleavage of the protruding loop is unlikely to alter the interaction between adjacent LRRs.

The interaction between TLR3N and TLR3C was found to be functionally important. TLR3N+C complex, not TLR3C alone, was able to sense dsRNA and activate both IFN-β and NF-κB promoters. Just 14-aa N-terminal deletion in TLR3 abolished TLR3N+C responses. Considering that the N-terminal portion of TLR3C locates in the LRRs, not the protruding loop, 14-aa deletion may prevent TLR3C association with TLR3N by altering the LRR structure. Similarly, the 14-aa extension of TLR3N im-

Immuno precipitation 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 ac-

Cell surface TLR3 expression on CD8+ DCs and MZ B cells was shown in the current study. Consistent with the results using in vitro cell lines (13, 21), cell surface expression of TLR3 on primary immune cells was dependent on Unc93B1. TLR3 was weakly detected on the J774 macrophage cell line and BM-MCs. TLR3 mAb PaT3 specifically enhanced TLR3 responses. This is not simply due to mAb-mediated TLR3 ligation, because PaT3 alone did not induce any activation. Moreover, only the mAb to TLR3N PaT3, but not CaT3, had the augmenting effect. Cell surface TLR3N may have a role in modulating TLR3 responses. Considering that PaT3 specifically augmented TLR3 responses, but not TLR4/MD-2 responses, PaT3 is likely to have a direct effect on TLR3 rather than inducing weak priming signal. PaT3 and CaT3 were both internalized, but still bound to TLR3. PaT3, but not CaT3, strengthened the association between TLR3N and TLR3C. PaT3-dependent augmentation of the association between TLR3N and TLR3C is likely to enhance TLR3 responses in endolysosomes, suggesting an important role of association be-

References


