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Distinct roles of TIR and non-TIR regions in the subcellular localization and signaling properties of MyD88

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Abstract MyD88 is a cytoplasmic adaptor protein that is critical for Toll-like receptor (TLR) signaling. The subcellular localization of MyD88 is characterized as large condensed forms in the cytoplasm. The mechanism and significance of this localization with respect to the signaling function, however, are currently unknown. Here, we demonstrate that MyD88 localization depends on the entire non-TIR region and that the correct cellular targeting of MyD88 is indispensable for its signaling function. The Toll-interleukin I receptor-resistance (TIR) domain does not determine the subcellular localization, but it mediates interaction with specific TLRs. These findings reveal distinct roles for the TIR and non-TIR regions in the subcellular localization and signaling properties of MyD88.

Keywords: MyD88; Non-TIR region; Signal transduction; Subcellular localization; TIR domain; TLR

1. Introduction

Toll-like receptors (TLRs) are key regulators in host defense mechanisms against infectious microorganisms [1]. More than 10 TLRs have been identified in mammals, all of which recognize highly conserved molecular structures in pathogens, ranging from bacterial cell-surface components to viral genomes. Ligand binding to a TLR initiates the recruitment of the Toll-interleukin I receptor-resistance (TIR) domain-containing adaptor proteins (TIR adaptors) to the cytoplasmic TIR domain of an activated TLR. TIR adaptors then initiate signaling events, eventually leading to the activation of the NF-κB, AP-1, and interferon regulatory factor (IRF) families of transcription factors, which induce the expression of genes involved in host defense against infection [2].

Four different TIR adaptors have been identified in mammals: MyD88, TIRAP/Mal, TRIF/TICAM1, and TRAM/TICAM2 [2]. MyD88 and TRIF play an essential role in downstream signaling, whereas TIRAP and TRAM may act to bridge the interaction of MyD88 and TRIF with specific TLRs. MyD88 is the first identified TIR adaptor that is essential for most TLR, IL-1 receptor, and IL-18 receptor signaling [3–7]. MyD88 is a protein of 296 amino acids and is composed of an N-terminal short region (N), a death domain (DD), an intermediate region (INT), and a C-terminal TIR domain. Previous research indicates that the TIR domain interacts with TLRs, whereas the DD is involved in downstream signaling. Several groups have reported the distinct subcellular localization of MyD88, which is characterized by condensed particles scattered throughout the cytosol [8–11]. Previous studies using CFP-YFP FRET analysis report a physical interaction between MyD88 and IRF-7 in these condensed particles [9,12]. Therefore, this distinct localization may be important for the signaling function of MyD88. However, the mechanism by which this localization is achieved is unknown, and its significance with respect to the function and structural features of MyD88 have not been studied in depth.

Each TIR adaptor interacts with specific TLRs and TIR adaptors. For example, MyD88 associates with all TLRs except TLR3, whereas TRIF only associates with TLR3 and TLR4. Furthermore, MyD88 and TRIF specifically interact with TIRAP and TRAM, respectively. There may be two factors that determine the specific partners for MyD88: a mechanical factor (e.g., specific TIR–TIR domain interaction) or a spatiotemporal factor (e.g., the distinct subcellular distributions of TLRs and TIR adaptors). However, the mechanism underlying the specificity of TLR–TIR adaptor and TIR adaptor–TIR adaptor interaction remains poorly understood.

Here, we report distinct roles for TIR and non-TIR regions with respect to the intracellular targeting of MyD88 and the interaction of MyD88 with specific TLRs and TIR adaptors. We found that the entire non-TIR region is responsible for the distinct subcellular localization of MyD88, and the correct cellular targeting of MyD88 is critical to its signaling function. In contrast, the TIR domain plays an essential role in determining the specificity of the MyD88–TLR interaction, whereas the specificity of the MyD88–TIRAP interaction was independent of the conformation of the TIR domain. These results not only describe the distinct roles of TIR and non-TIR regions in the subcellular localization and signaling properties of MyD88, they also provide evidence that the diversity of TLR signaling may be achieved by both TIR domain-dependent and -independent mechanisms.

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Abbreviations: CFP, cyan fluorescent protein; CTXb, cholera toxin subunit b; DD, death domain; TIR, Toll-interleukin I receptor-resistance; TLR, Toll-like receptor; YFP, yellow fluorescent protein; INT, Intermediate region

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2. Materials and methods

2.1. Reagents and cell cultures

Flagellin and CpG-B oligodeoxynucleotides [13] were purchased from InvivoGen (San Diego, CA, USA) and Sigma Genosys (Ishikari, Japan), respectively. Anti-α-tubulin antibody, Alexa Fluor 594- and 647-conjugated cholera toxin subunit B (CTXb), Alexa Fluor 546-conjugated anti-mouse IgG, Alexa Fluor 594-conjugated phalloidin, rhodamine B-conjugated dextran (MW 10 000), and LysoTracker Red DND-99 were purchased from Invitrogen (Carlsbad, CA, USA). Anti-KDEL antibody and anti-58K Golgi protein antibody were purchased from Stressgen (Ann Arbor, MI, USA) and Abcam (Cambridge, UK), respectively. Anti-GFP antibody (clone JL-8) and NF-κB luciferase reporter construct were purchased from Clontech (Mountain View, CA, USA). Anti-HA antibody and anti-FLAG(M2) peroxidase conjugate were purchased from Covance (Berkeley, CA, USA) and Sigma (St. Louis, MO, USA), respectively.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). All HEK293T stable transformants were prepared by retroviral gene transfer as described previously [14].

2.2. DNA constructs

The TIR domain-swap mutant (TRIFTIR) was constructed by "PCR sewing" of cDNA corresponding to amino acids 1–160 of mouse MyD88 and amino acids 397–534 of mouse TRIF. The resulting cDNA was cloned into the pMXpie-N-HA retroviral vector. The cDNAs encoding HA-tagged mouse MyD88, HA-tagged TRIFTIR, and FLAG-tagged mouse TIRAP were subcloned into the pMXpie retroviral vector [15] and pEF-BOS-EX vector (a kind gift from Dr. Shigekazu Nagata, Kyoto University [16]). The C-terminal yellow fluorescent protein (YFP)- or cyan fluorescent protein (CFP)-tagged full-length MyD88 (amino acids 1–296), N (amino acids 1–31), N–DD (amino acids 1–109), N–DD–INT (amino acids 1–160), DD–INT–TIR (amino acids 32–296), Δ(1–51) (amino acids 52–296), INT–TIR (amino acids 110–296), TRIFTIR, TLR5, and TLR9 were constructed by subcloning cDNA encoding each protein into pMXrmv5-$(G_4S)_3$YFP or pMXrmv5-$(G_4S)_3$CFP retroviral vectors. YFP or CFP was fused to the C-terminus of the protein of interest via a short linker sequence [(Gly-Gly-Gly-Gly-Ser) × 3] within the vectors. YFP-tagged TLR4 was described previously [17].

2.3. Microscopy

Cell-surface staining with CTXb was performed as described previously [18]. A Z-Stack image was collected using a KEYENCE BZ-9000 fluorescent microscope with step sizes of 0.2 μm.

For the staining of various subcellular markers, cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen, San Diego, CA, USA) and then treated with anti-α-tubulin antibody (1:200 dilution), anti-KDEL antibody (1:200 dilution), or anti-58K Golgi protein antibody (1:250 dilution) for 1 h at 4 °C, followed by Alexa Fluor 546-conjugated anti-mouse IgG antibody (1:200 dilution) for 30 min at 4 °C or Alexa Fluor 594-conjugated phalloidin (1:40 dilution) for 20 min at room temperature. For endosome staining, cells were treated with 1 mg/ml rhodamine B-conjugated dextran for 10 min (early endosome) or 2 h (late endosome) at 37 °C [19]. For lysosome staining, cells were treated with LysoTracker Red DND-99 (1:20 000 dilution) for 30 min at 37 °C. The images were acquired using a Bio-Rad MRC1024 laser scanning confocal microscope.

2.4. Gel filtration analysis

HEK293T cells stably expressing MyD88-YFP (HEK293T-MyD88-YFP, 1×10^7) were lysed with 500 μl of lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and complete protease inhibitor cocktail [Roche, Mannheim, Germany], pH 7.4). The lysate was centrifuged, and the supernatant was loaded onto a Superose 6 HR10/30 column (GE Healthcare, Little Chalfont, UK). Fractions (0.5 ml) were analyzed by immunoblotting using anti-GFP antibody. The apparent molecular weight was evaluated after column calibration with protein standards: thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), and ovalbumin (43 kDa).

2.5. Luciferase reporter assay

HEK293T cells were plated at a density of 2×10^5 cells/well in a 24-well plate. One day after plating, the cells were transiently transfected with 10 ng of NF-κB luciferase reporter plasmid together with 0.8 μg of retroviral expression plasmid(s). Luciferase activity in the total cell lysate was monitored for 24–48 h after transfection using the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

2.6. Co-imunoprecipitation

Cells (2×10^6) were transiently transfected with 8 μg of pEF-BOS-EX-based expression plasmid(s). Twenty-four hours after transfection, cells were lysed with 600 μl of lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, and protease inhibitor cocktail [Roche], pH 7.5). The supernatants were incubated with anti-GFP antibody for 16 h at 4 °C. Protein G-Sepharose 4 Fast Flow (GE Healthcare) was added and the samples were incubated for 4 h at 4 °C. The beads were washed four times with 1 ml of lysis buffer, boiled with SDS sample buffer, and subjected to immunoblotting using the indicated antibodies.

2.7. GST pull-down assay

GST fusion proteins were expressed in BL21-Gold bacteria by 3 h of induction with 0.1 mM IPTG (isopropyl-β-D(−)-thiogalactopyranoside). Bacterial pellets were resuspended in buffer A, consisting of 5 mM dithiothreitol (DTT) and 1% Triton X-100 in phosphate-buffered saline (PBS; pH 7.4). After sonication, the lysates were centrifuged and the supernatants were incubated with Glutathione-Sepharose 4B (GE Healthcare) for 1 h at 4 °C. The beads were washed five times with 1 ml of buffer A and resuspended in lysis buffer to prepare a 50% slurry.

HEK293TCM cells [17] stably expressing TLR4-YFP (HEK293TCM-TLR4-YFP; 6×10^6) were transfected with 24 μg of empty vector or pEF-BOS-EX-TIRAP-FLAG. After 48 h, lysates were prepared, and the supernatants were incubated with 10 μg of GST fusion proteins for 1 h at 4 °C. The GST fusion protein-bound beads were washed three times with lysis buffer, boiled with SDS sample buffer, and subjected to immunoblotting using anti-FLAG antibody.

3. Results and discussion

3.1. MyD88 is localized in the cytoplasm in condensed, morphologically diverse forms

Previous studies have shown that MyD88 is present in discrete foci scattered throughout the cytosol [9–11]. We also observed this distinct characteristic using MyD88-YFP fusion proteins in HEK293T cells (Fig. 1A) and bone marrow-derived macrophages (data not shown). MyD88-YFP induced NF-κB activation when over-expressed (Fig. 1B) and mediated TLR signaling in a ligand-dependent manner (Fig. 5A), indicating that the MyD88-YFP fusion protein is functional. The Z-Stack image collected from wide-field microscopy showed that the MyD88-YFP signals were morphologically diverse (Fig. 1C), belonging to one of five typical morphologies: spot, star, string, ring, and cocoon (Fig. 1D). The over-expressed proteins are sometimes misfolded and form aggregates in the cytoplasm, causing improper subcellular distribution. To rule out the possibility that this distinct subcellular localization of MyD88-YFP was an artifact of over-expression, we performed gel filtration analysis to fractionate lysates from HEK293T-MyD88-YFP cells based on molecular size. MyD88-YFP, which has a calculated molecular size of 61.8 kDa, was detected in fractions numbers. 32 and 33, corresponding to 67 kDa and 132 kDa, respectively (Fig. 1E). These data indicate that MyD88-YFP is present as a monomer and/or homodimer in the cytoplasm, which is consistent with earlier observations by Burns et al. [5], who found that MyD88 forms

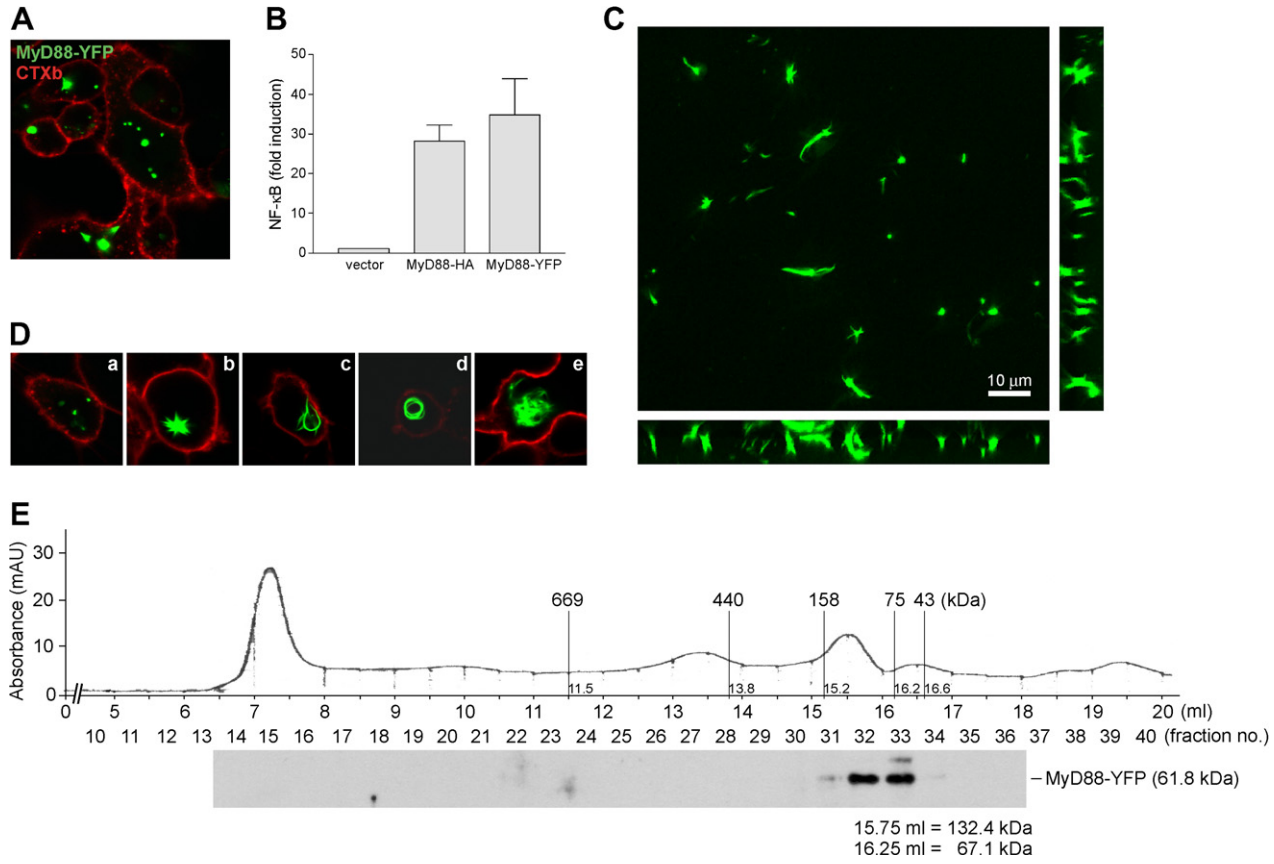

Fig. 1. MyD88 is localized in the cytoplasm as large condensed forms. (A) Confocal image of HEK293T-MyD88-YFP cells stained with Alexa Fluor 594-conjugated CTXb. (B) HEK293T cells were transiently transfected with 0.8 μg of the indicated expression plasmids along with 10 ng of NF-κB reporter construct. Luciferase activity was measured 24 h after transfection. Results are shown as the means ± standard deviation of three independent experiments. (C) Z-Stack image of MyD88-YFP in HEK293T cells. (D) Typical morphologies of MyD88-YFP in HEK293T cells: (a) spot; (b) star; (c) string; (d), ring; (e), cocoon. (E) HEK293T-MyD88-YFP cells were lysed and fractionated on a Superose 6 gel filtration column. MyD88-YFP was analyzed by immunoblotting using anti-GFP antibody. The elution positions of molecular weight markers (in kDa) and the protein concentration of the eluent monitored in real-time (mAU) are indicated.

a homodimer. Therefore, we concluded that the unusual subcellular localization of MyD88-YFP is not an artifact, but instead a characteristic of endogenous MyD88.

3.2. MyD88 localizes to specific intracellular organelles

Our data from microscopy and gel filtration analysis suggest that MyD88 is present in vesicle-like structures or associated with tubular or fibrillar structures. To identify the intracellular compartments in which MyD88 is enriched, HEK293T-MyD88-YFP cells were stained with markers for the endoplasmic reticulum (ER), Golgi apparatus, endosomes, and lysosomes. This experiment was based on observations that some of the morphologies of MyD88-YFP are similar to the morphologies of these organelles; in addition, TLRs have been identified in these compartments [20–23]. We used the following markers: proteins containing a C-terminal KDEL sequence for the ER, 58K Golgi proteins for the Golgi apparatus, dextran for early and late endosomes, and LysoTracker for lysosomes. However, no clear co-localization of MyD88-YFP and these markers was observed (Fig. 2). We also examined the co-localization of MyD88 with cytoskeletal proteins such as F-actin and α-tubulin. This was based on observations that some of the morphologies of MyD88-YFP appeared similar to those of the cytoskeletal structures; furthermore, MyD88 is associated with fibrillar aggregates containing β-actin in the cytoplasm [8]. However, neither F-actin (phalloidin staining) nor α-tubulin was clearly co-localized with MyD88-YFP (Fig. 2). These results suggest that MyD88 resides in as yet uncharacterized organelles.

3.3. The entire non-TIR region is required for the distinct subcellular localization of MyD88

Typical sequences for targeting proteins to intracellular compartments, such as the KDEL sequence in ER proteins, have not been identified in MyD88. The primary structure of MyD88 can be divided into four regions: the N-terminal short region (N), a death domain (DD), an intermediate region (INT), and a TIR domain [5]. Therefore, we investigated which regions are involved in the subcellular localization of MyD88. We generated several truncated mutants of MyD88 and a TIR domain-swap mutant (TRIFTIR) in which the TIR domain of MyD88 was replaced with the corresponding region of TRIF [24,25] (Fig. 3A). The mutant proteins fused with YFP at the C-terminus were expressed in HEK293T cells, and the subcellular localization was examined under a microscope. The TRIFTIR mutant and the TIR domain-deleted mutant (N–DD–INT) were localized in the cytoplasm in condensed form (Fig. 3B). Furthermore, these mutants were clearly co-local-

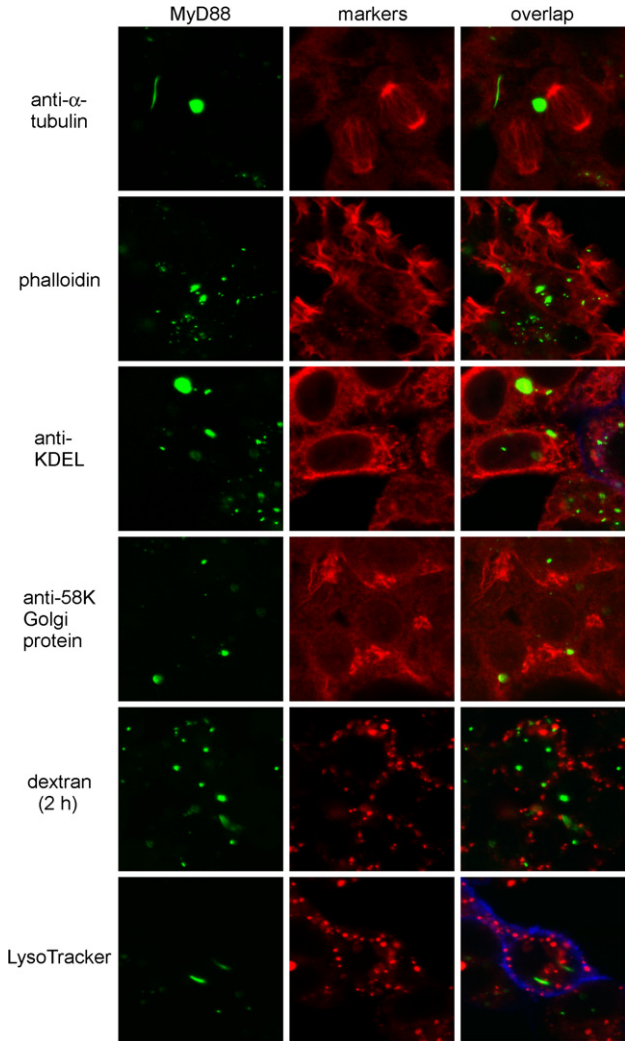

Fig. 2. MyD88 resides in uncharacterized organelles in HEK293T cells. HEK293T-MyD88-YFP cells were stained with the indicated markers as described in Section 2. For LysoTracker staining, the cells were counterstained with CTXb to visualize the cell surface [blue (pseudocolor)].

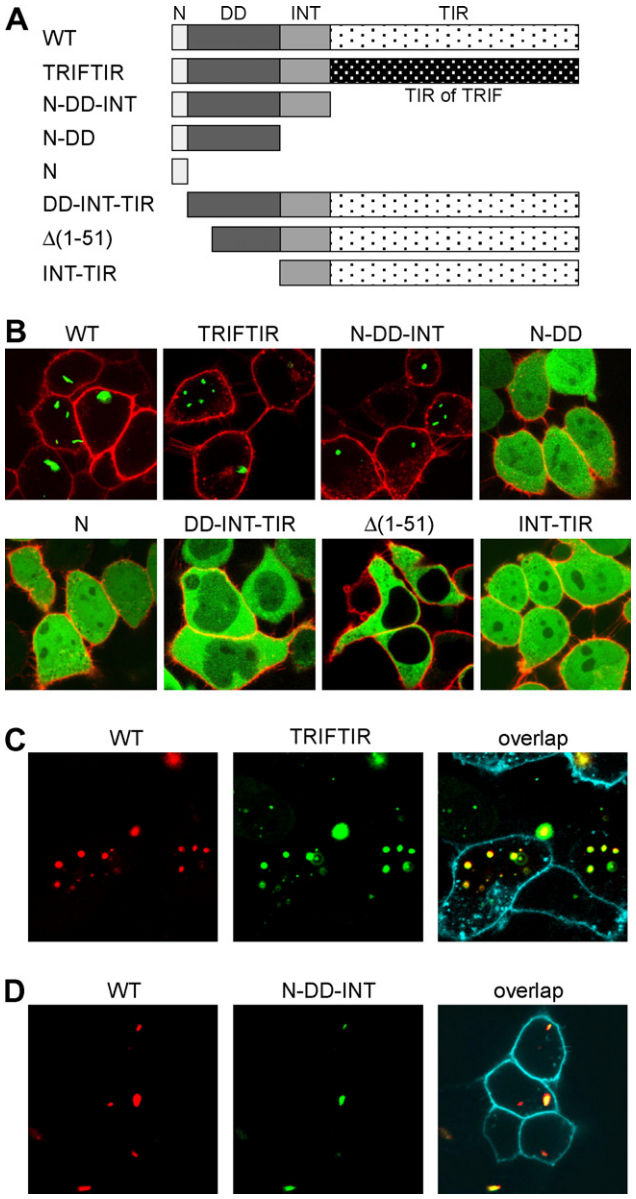

Fig. 3. The entire non-TIR region is responsible for the distinct subcellular localization of MyD88. (A) Schematic diagram of MyD88 mutant proteins. (B) Confocal images of HEK293T cells expressing the proteins depicted in A. The images were created as described in Fig. 1A. MyD88 and its mutant proteins are shown in green; CTXb is shown in red. (C, D) Confocal images of HEK293T cells expressing MyD88-CFP and TRIFTIR-YFP (C) or N–DD–INT–YFP (D). The images were created as described in Fig. 1A. MyD88-CFP is shown in red (pseudocolor); TRIFTIR-YFP and N–DD–INT–YFP are shown in green; CTXb is shown is light blue (pseudocolor); co-localized signals appear yellow.

ized with wild-type MyD88 in the cytoplasm (Fig. 3C and D). In contrast, all other mutants lacking a part of the non-TIR region were diffusely expressed throughout the cytoplasm and/or nucleus. These results suggest that the entire non-TIR region plays a critical role in localizing MyD88 within the cell.

3.4. Correct cellular targeting of MyD88 is critical to the activation of signaling events

The functional significance of this distinct subcellular localization is unclear. MyD88 associates with downstream signaling molecules such as interleukin-1 receptor-associated kinase 1 (IRAK1) [3] and IRF-7 [12] through its DD. Therefore, we used DD-containing MyD88 mutants to examine the significance of the subcellular localization in signal transduction. MyD88 and its mutants were over-expressed in HEK293T cells, and NF-κB activity was measured using a reporter assay. Interestingly, the TRIFTIR and N–DD–INT mutants that were co-localized with wild-type MyD88 induced NF-κB activation, whereas the N-DD and DD–INT–TIR mutants that were diffusely expressed throughout the cytoplasm and nucleus did not (Fig. 4). These results suggest that the correct cellular localization is essential for the activation of signaling events.

3.5. The specificity of TLR-MyD88 interaction is determined by the conformational characteristics of the TIR domain

Each TLR interacts with specific TIR adaptors, allowing the innate immune system to induce distinct physiological

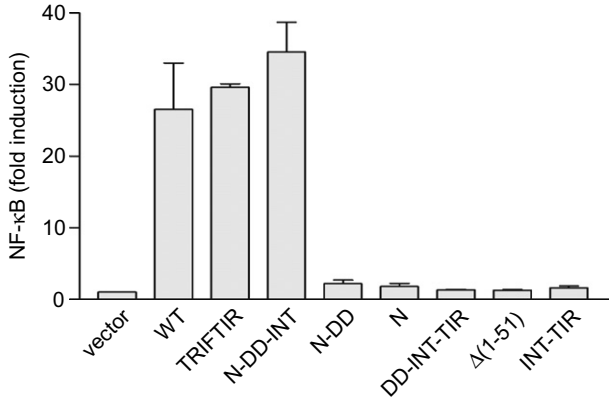

Fig. 4. Correlation between subcellular localization of MyD88 and its signaling function. HEK293T cells were transfected with 0.8 μg of expression plasmids encoding the proteins depicted in Fig. 3A along with 10 ng of NF-κB reporter construct. Luciferase activity was measured 24 h after transfection. Results are shown as the means ± standard deviation of three independent experiments.

responses to distinct infectious microorganisms [26]. There are two factors that may determine the specificity of the TLR–TIR adaptor interaction: the conformational characteristics of each TIR domain (i.e., a mechanical factor) and the distinct subcellular localizations of TLRs and TIR adaptors (i.e., a spatiotemporal factor). Both TLR5 and TLR9 signaling are totally dependent on MyD88, but not other TIR adaptors [2]. To identify the mechanism that determines the interaction specificity between these proteins, wild-type MyD88 and the TRIFTIR mutant were co-expressed with TLR5 or TLR9 in HEK293T cells. The cells were stimulated with flagellin for TLR5 or CpG-B for TLR9, and NF-κB activity was measured using a reporter assay. Ligand-dependent NF-κB activation was observed in the cells expressing MyD88, but not in those expressing the TRIFTIR mutant, although wild-type MyD88 and the TRIFTIR mutant show similar constitutive activities (Fig. 5A). Given that the TRIFTIR mutant co-localizes with wild-type MyD88, these results suggest that the specificity of the interaction between MyD88 and TLR5 or TLR9 is determined by the conformational characteristics of each TIR domain; the TIR domain of MyD88 has a high affinity for TLR5/9, whereas the TIR domain of TRIF has low affinity.

We performed co-immunoprecipitation analysis to examine the physical interaction between TLR and MyD88. Consistent with the results from the NF-κB reporter assay, MyD88 co-immunoprecipitated with TLR5 in a ligand-dependent manner, whereas the TRIFTIR mutant did not co-immunoprecipitate (Fig. 5B). These results demonstrate that the specificity of the TLR–MyD88 interaction is determined by the conformational characteristics of each TIR domain, rather than the spatiotemporal characteristics of the subcellular localization of TLRs and MyD88.

3.6. The specificity of the MyD88–TIRAP interaction is independent of the conformational characteristics of the TIR domain

The association of MyD88 with TLR4 is distinct from its association with TLR5 or TLR9. TLR4 requires another TIR adaptor, TIRAP, to associate with MyD88 and activate the MyD88-dependent signaling pathway [27,28]. TIRAP specifically associates with MyD88 via TIR–TIR interaction and facilitates the delivery of MyD88 to activated TLR4 [11]. Because TIRAP forms a heterodimer with MyD88 [27,28], we expected that the specificity of the MyD88–TIRAP interaction is also determined by the conformational characteristics of each TIR domain. To test this assumption, the effect of wild-type MyD88 and the TRIFTIR mutant on TLR4-induced NF-κB activation was examined. HEK293T cells stably expressing CD14 and MD-2 (HEK293TCM cells [17]) were transiently transfected with TLR4 and wild-type MyD88 or TRIFTIR mutant, and then NF-κB activity was measured using a reporter assay. Surprisingly, TLR4 and the TRIFTIR mutant synergistically induced NF-κB activation; the activity in cells co-expressing both TLR4 and the TRIFTIR mutant was much higher (21.7 ± 2.2-fold increase) than the activity estimated by summing the values from cells expressing TLR4 alone and cells expressing TRIFTIR alone

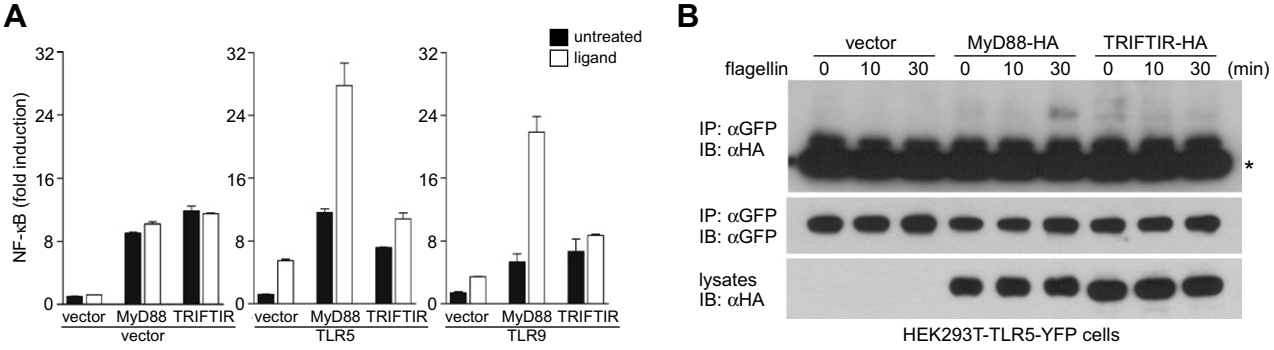

Fig. 5. The interaction of MyD88 with specific TLRs is dependent on the conformational characteristics of the TIR domain. (A) HEK293T cells were co-transfected with 10 ng of NF-κB reporter construct and the expression plasmids for the indicated combinations of empty vector (0.1 or 0.7 μg), MyD88 (0.1 μg), TRIFTIR (0.1 μg), TLR5-YFP (0.7 μg), and TLR9-YFP (0.7 μg). After 24 h, the cells were treated with 100 ng/ml flagellin for vector and TLR5 or 5 μM CpG-B for TLR9 and further incubated for 16 h at 37 °C. NF-κB-dependent luciferase activity was then analyzed. Results are shown as the means ± standard deviation of three independent experiments. (B) HEK293T cells stably expressing TLR5-YFP (HEK293T-TLR5-YFP) were transfected with empty vector, MyD88-HA, or TRIFTIR-HA. After 24 h, the cells were stimulated with 100 ng/ml flagellin at the indicated times. Lysates were prepared, and co-immunoprecipitation analysis was performed as described in Section 2. Lysates were analyzed to confirm the equivalent expression of the appropriate proteins in each sample. The asterisk next to the upper panel shows the immunoglobulin light chain of anti-GFP antibody used in the immunoprecipitation.

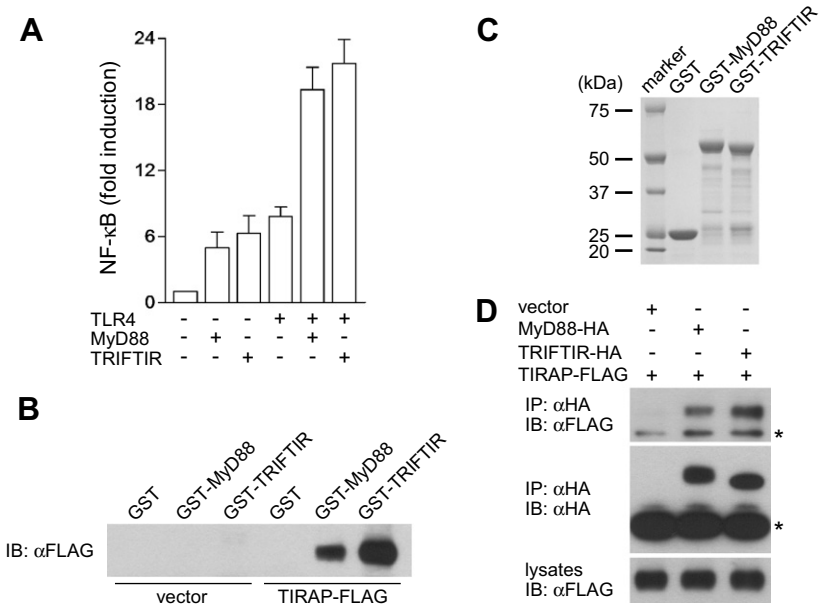

Fig. 6. The specific interaction of MyD88 with TIRAP is independent of the conformational characteristics of the TIR domain. (A) HEK293TCM cells [17] were co-transfected with 10 ng of NF-κB reporter construct and the expression plasmids for the indicated combinations of empty vector (0.7 or 0.8 μg), MyD88 (0.1 μg), TRIFTIR (0.1 μg), and TLR4-YFP (2 ng). After 24 h, NF-κB-dependent luciferase activity was measured. Results are shown as the means ± standard deviation of three independent experiments. (B) GST pull-down assay. GST fusion proteins were incubated with lysates prepared from HEK293T cells transfected with empty vector or TIRAP-FLAG. The interaction of TIRAP with GST fusion proteins was detected by immunoblotting with anti-FLAG antibody. (C) Coomassie blue staining to detect GST-MyD88, GST-TRIFTIR, and GST proteins purified from bacteria. (D) Lysates from 293TCM-TLR4-YFP cells transfected with the indicated expression plasmids were subjected to co-immunoprecipitation analysis as described in Fig. 5. The asterisks next to the upper and the middle panels show the immunoglobulin light chain of anti-HA antibody used in the immunoprecipitation.

(14.1 ± 2.4-fold increase). These results suggest that the TRIFTIR mutant mediates TLR4 signaling. To test whether the TRIFTIR mutant is required to physically interact with TIRAP to mediate TLR4 signaling, we performed a GST pull-down assay. TIRAP interacted with the GST-TRIFTIR mutant more efficiently than with the GST-MyD88 proteins (Fig. 6B). Coomassie blue staining confirmed that the same amounts of GST-fusion proteins were used in the assay (Fig. 6C). Furthermore, we investigated the interaction of TIRAP and the TRIFTIR mutant using co-immunoprecipitation analysis. Consistent with the results from the GST pull-down assay, TIRAP co-immunoprecipitated more efficiently with the TRIFTIR mutant than with wild-type MyD88 in HEK293TCM-TLR4-YFP cells (Fig. 6D). These results suggest that the affinity of the TIR domain of TRIF for TIRAP may be stronger than that for MyD88. It is likely that the MyD88–TIRAP interaction required for TLR4 signaling is independent of the conformational characteristics of the TIR domain.

Although our results showed a physical interaction between the TRIFTIR mutant and TIRAP, TRIF itself does not interact with TIRAP. Similar to TLRs, the TIR adaptors exhibit a notably diverse subcellular distribution. TRIF is diffusely expressed in the cytoplasm [12], whereas TIRAP and TRAM are localized on or proximal to the plasma membrane owing to their targeting motifs, which include phosphatidylinositol 4,5-bisphosphate (PIP2)-binding domains and myristoylation sites [11,29]. Because the TRIFTIR mutant was co-localized with MyD88 (Fig. 3C), TRIF might interact with TIRAP if TRIF is targeted to a location in which MyD88 is enriched.

3.7. Concluding remarks

MyD88 is present in condensed, morphologically diverse forms in the cytoplasm. Our results demonstrate that the entire non-TIR region is sufficient for targeting MyD88 to as yet uncharacterized locations within the cell, where it interacts with signaling molecules and induces host defense responses. Furthermore, the interaction between MyD88 and specific TLRs is likely determined by the conformational characteristics of each TIR domain, whereas the specific interaction of MyD88 and TIRAP required for TLR4 signaling is likely determined by other spatiotemporal factors. These distinctive properties of the TIR and non-TIR regions are important for inducing effective immune responses via targeting MyD88 to the correct cellular location and by preventing MyD88 from associating with inappropriate partners.

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