

Biophysical analysis of MyD88 and related proteins

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Preface

This thesis reflects the author's studies and discussions as the Department of Molecular Engineering, Graduate School of Engineering, Kyoto University, during the period from April 2014 to March 2019. The purpose of this thesis is to elucidate the molecular mechanisms underlying the innate immune system centered on an adaptor protein called MyD88

Chapter II provides an analysis of the intramolecular interaction between two domains of MyD88. MyD88 comprises two functional domains; N-terminal kinase interacting module called DD_{MyD88} (death domain) and C-terminal receptor interacting module called TIR_{MyD88} (Toll/IL-1 receptors domain), and the main function of each domain is well understood. However, their relative positions have not been eagerly investigated. This chapter proposes a novel regulatory mechanism of the activity of MyD88 based on the interaction between DD_{MyD88} and TIR_{MyD88} by using NMR and high-speed AFM. The experiments identify the interaction site of for intramolecular interaction, and show the dynamic equilibrium between open- and closed-state of full-length MyD88, which may correspond to active- and resting-state, respectively.

Chapter III provides the structural basis of self-assembled complexes of TIR_{MyD88}. TIR_{MyD88} is known to form homo-dimers, whereas DD_{MyD88} assembles into helical homo-oligomers. However, this chapter shows that TIR_{MyD88} assembled into a large annular oligomeric complexes by using high-speed AFM. The complex is constituted from 58 TIR_{MyD88} molecules with a 29-fold rotational symmetric bilayer ring. Further cryo-EM analysis demonstrates that the domain forms tube-like filaments in solution, each of which comprises multitude of bilayer rings stacked tandemly.

Chapter IV provides a molecular analysis of inactivation mechanism of human TRPC6. Previous reports suggested calmodulin contributed to the TRPC6 inactivation, but the molecular details are largely unknown. This chapter demonstrates that calmodulin binds a short peptide derived from TRPC6 in 1:2 stoichiometry, with which a novel molecular mechanism for the TRPC6 inactivation by calmodulin is proposed.

Chapter V provides a summary with a general discussion of the author's studies.

Chapter I

General Introduction

1. Innate immunity and adaptive immunity

All living organisms have the immune system to defend themselves against invaders from the surroundings, which has been developed as a complicated mechanism during the course of evolution. In general, the immune system is broadly classified into two categories; innate immunity and adaptive immunity.

The innate immune system functions as the early response of the whole immune system¹. Leucocytes such as macrophages, dendritic cells, and neutrophils and their functional networks constitute an important arm of the innate immune system, and their functions are diverse; pathogen recognition, immune system activation, inflammation induction, phagocytosis, and antigen presentation². Among these functions, pathogen recognition is particularly important, because it is the first response of the immune system against any infection. PRRs (pattern recognition receptors) are the receptor family responsible for this response. They recognize PAMPs (pathogen-associated molecular patterns), constituent molecules of pathogens, such as LPS (lipopolysaccharide), lipoprotein, flagellin, and viral nucleotide³. Once they detect PAMPs, PRRs induce production of cytokines and chemokines etc. via the intracellular signaling, which stimulate the immune systems to evoke immune responses.

The adaptive immune system is characterized by a pathogen-specific response mediated via antibodies and T-cell receptors. Lymphocytes such as B-cells and T-cells and their associated functional networks are important constituents of the adaptive immune system⁴. B-cells produce antibody molecules based on information presented by phagocytes via helper T-cells, and the produced antibody molecules have a specific interaction site for the pathogens that were degraded and presented by the phagocytes². After antibody molecules bind the surface antigens of the pathogens, cytotoxic T-cells and phagocytes attack on the pathogens interacting with antibody molecules. Moreover, memory B-cells preserve the antigen information presented by phagocytes, which allows rapid immune responses against re-invasion of the same pathogens in future. When compared, the innate immunity is superior regarding its rapidity and versatility, whereas the adaptive immunity is excellent regarding its efficiency and specificity. These different characters of the two systems contribute to the high adaptability of the immune system in combating against numerous and diverse pathogens.

2. Inflammation

Inflammation is one of homeostatic response against any deviation from homeostasis including infection, and it is anatomically characterized by four symptoms, namely, fever, pain, redness and swelling². Inflammation is a typical response in the innate immune system, and it takes various roles in the early immune response. Generally, the early immune response is constituted from a four-step sequential process; firstly, the secretion of inflammatory cytokines from immunocytes that recognized PAMPs or DAMPs (damage-associated molecular patterns), secondly, the deformation of blood vessels and enlargement of blood flow in order to accumulate immunocytes into affected area, thirdly, leucocytes activation and accumulation in affected area, finally, full operation of the immune system. Anatomical observations reflect a part of these responses.

When the cause of the deviation from homeostasis is eliminated, the inflammation gradually comes to an end. This transient case is known as acute inflammation, and it is a physiological response. However, when something inhibits convergence of inflammation, the transient inflammation shifts to a pathogenic state called as chronic inflammation⁵. In chronic inflammation, the constitutive activation of the immunocyte including cytotoxic cells leads to the attack against host organisms, causing intractable autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and type-I diabetes⁶⁻⁸.

3. Cytokine network

Cytokines are a broad category of small secreted proteins related in various intercellular signal transductions. Especially, the immune system used various cytokines. However, not only immunocytes but also other type of cells secreted cytokines, and cytokines affect various cells⁹. One of the most important functions of cytokines is regulation of immune responses in different phases of both innate and adaptive immunity¹.

Generally, a cytokine affects the immune system based on a series of sequential interactions known as the cytokine network. The cytokine network mainly comprises three cyclic processes, (1) a receptor binds a secreted cytokine, (2) the ligand-bound receptor activates transcriptional factors via intracellular signal transduction³, (3) the transcriptional factors induce immune responses including expression and secretion of further cytokines, (4) secreted cytokines repeat the cycle from (1). Based on cytokines recognition and production in step (1) and (3), immunocytes control other cells (Fig.1a)¹⁰⁻¹⁴. Moreover, cytokines affect the activity of other cytokines by signal crosstalk caused by the overlap of proteins used for multiple intracellular signal transduction pathways (Fig. 1b)^{3,15}. These bidirectional intercellular and intracellular interactions constitute the cytokine network and harmonically regulate immune responses. Because the cytokine network functions as the central regulatory system of the immune system, its dysregulation can cause various diseases, such as auto-immune diseases, immune deficiency, septicemia, and lymphoma.

Most of immune-related cytokines are classified into IL (interleukin) family, TNF (tumor necrosis factor) family, GF (growth factor) family, IFN (interferons), and chemokines. This classification is based on molecular structures, but the classification and function of each member do not always match (Table 1)¹⁶. Among them, cytokines related in the exacerbation of inflammation known as inflammatory cytokines have been drawing attention as therapeutic drug targets, because some inflammatory cytokines play essential roles in the pathomechanism of intractable diseases⁹. For instance, TNF- α is known as a representative inflammatory cytokine and has been studied as a treatment target in rheumatoid arthritis^{7,17}. The effort developed a number of anti-TNF- α antibody drugs for treating rheumatoid arthritis, such as Infliximab, Adalimumab, and Golimumab¹⁸.

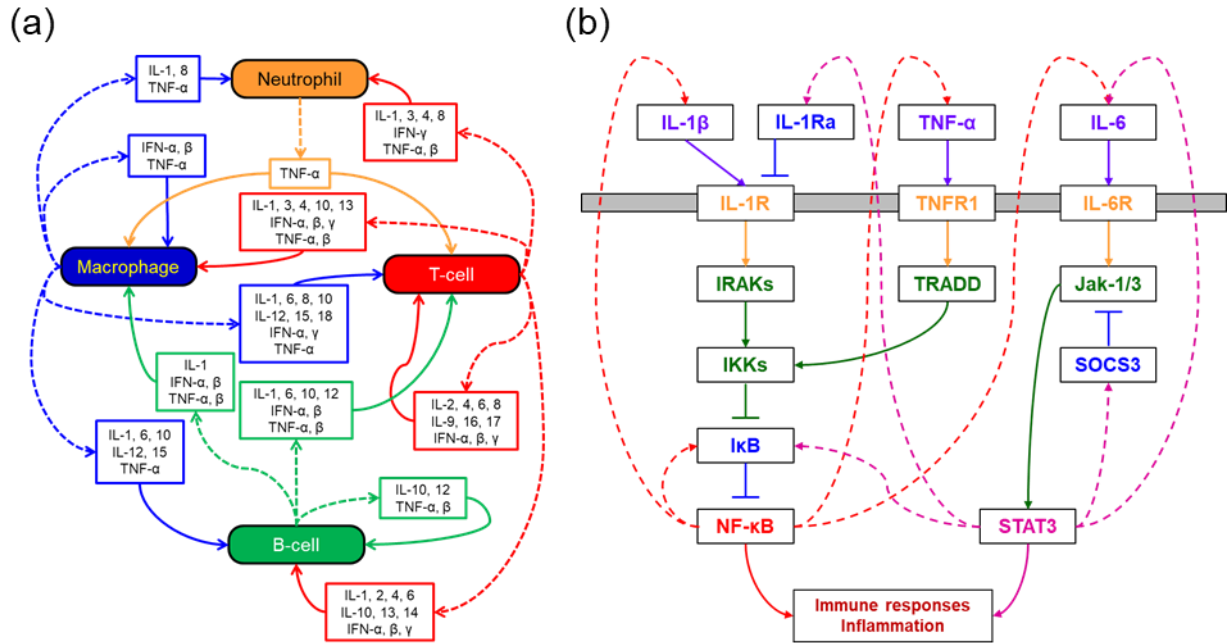


Figure 1 Schematic diagram of the cytokine network.

(a) A schematic diagram of the cytokine network of immunocytes, such as neutrophils, macrophages, T-cells and B-cells^{10–14}. Rectangle, rounded rectangle, arrow, and broken arrow correspond to secreted cytokines, immunocytes, affection, and secretion, respectively. Immunocytes regulate each other's state via secretion and reception of cytokines. (b) A schematic diagram for the signal crosstalk in inflammatory cytokine signals^{3,15}. Arrow, broken arrow, and T-bar correspond to activation, expression, and inhibition, respectively. Intracellular signals triggered by inflammatory cytokines such as IL-1β, TNF-α and IL-6 interact with each other and regulate harmonically.

Table 1 Summary of cytokine classification based on function¹⁶.

Function	Representative Cytokines
Inflammation	IL-1β, IL-6, TNF-α
Antiinflammation	IL-10, IFN-α/β, TGF-β
Immunocyte migration	IL-8, CCL1, CXCL6
Immunocyte activation	IL-3, IL-12, IFN-γ
Immunocyte differentiation	IL-2, IL-4, IL-5, IL-7
Cell Growth	EGF, TGF-β, G-CSF
Apoptosis	TNF-α, TRAIL, N-APP

4. Pattern recognition receptors

PRRs (Pattern recognition receptors) are a receptor family that recognizes PAMPs and DAMPs. PRRs are classified into TLRs (Toll-like receptors), NLRs (Nod-like receptors), ALRs (AIM2-like receptors), RLRs (RIG-I-like receptor) and CLRs (C-type lectin receptor)¹⁹. These receptors are localized in the plasma membrane, endosome, or cytosol. Each receptor widely recognizes each corresponding molecular species, PAMPs or DAMPs (Table 2)^{19–25}. Multitude of different PRRs recognize different ligand molecules. However, there is a clear relationship between target pathogens and activated proteins, because intracellular signal transduction pathways activated by multiple PRRs are converged in the activation of some specific proteins. Specifically, PRRs corresponded to viral component molecules activate transcriptional factors called IRF-3/7 (interferon regulatory factor 3 and 7), which produce anti-viral cytokines and interferons. On the other hand, PRRs corresponded to bacterial and fungal component molecules mainly activate a transcriptional factor called NF- κ B (nuclear factor- κ B), which produces inflammatory cytokines widely. Some PRRs activate caspase-1, which executes maturation of immature form of inflammatory cytokines. Moreover, there are some cases wherein a ligand molecule is recognized by multiple PRRs localized at different regions, such as TLR5 and NLRC4. Based on such diversity and redundancy, PRRs recognize various pathogens widely and certainly.

Table 2 Summary of PRRs^{19–25}.

Family	Receptor	Ligand	Localization	Target pathogen	Activated protein
TLRs	TLR1/2	Triacyl lipoproteins	Plasma membrane	Bacteria	NF- κ B
	TLR2/6	Diacyl lipoproteins	Plasma membrane	Bacteria	NF- κ B
	TLR5	Fragelin	Plasma membrane	Bacteria	NF- κ B
	TLR4	Lipopolysuccaride	Plasma membrane	Bacteria	NF- κ B
		Lipopolysuccaride	Endosome	Virus	IRF-3/7
	TLR3	double strand RNA	Endosome	Virus	IRF-3/7
	TLR7/8	single strand RNA	Endosome	Virus	IRF-3/7
	TLR9	CpG methylated DNA	Endosome	Virus	IRF-3/7
NLRs	NOD1	Peptidoglican	Cytosol	Bacteria	NF- κ B
	NLRC4	Fragelin	Cytosol	Bacteria	Caspase-1
	NLRP3	Crystal	Cytosol	DAMPs	Caspase-1
ALRs	AIM2	double strand DNA	Cytosol	Virus	Caspase-1
	IFI16	double strand DNA	Cytosol	Virus	IRF-3/7
	MNDA	double strand DNA	Cytosol	Virus	IRF-3/7
RLRs	RIG-I	double strand RNA	Cytosol	Virus	IRF-3/7
	MDA5	double strand RNA	Cytosol	Virus	IRF-3/7
	LGP2	double strand RNA	Cytosol	Virus	IRF-3/7
CLRs	Dectin-1	β -Glucan	Plasma membrane	Fungus	NF- κ B
	Dectin-2	β -Glucan	Plasma membrane	Fungus	NF- κ B
	Mincle	SAP130	Plasma membrane	Fungus	NF- κ B

5. NF- κ B activation via the MyD88-dependent canonical pathway

NF- κ B is a transcriptional factor that takes a central role in the inflammatory cytokine network²⁶. NF- κ B is activated by intracellular signals from various receptors, such as PRRs, inflammatory cytokines receptors, growth factor receptors, B-cell receptors, and T-cell receptors to produce various inflammatory cytokines²⁷. Therefore, the intramolecular signal transduction pathway to NF- κ B is one of the most critical pathways in early immune responses and inflammation²⁶.

In various pathways to NF- κ B, the MyD88 (Myeloid differentiation factor 88)-dependent canonical pathway is the most essential signaling pathway, which is activated at the earliest stage of the immune response. Receptors that can activate the pathway are IL-1fRs (interleukin-1 family protein receptors) and most of TLRs (Fig.2)^{28,29}. In the pathway, ligand-bound IL-1fRs and TLRs first interact with MyD88, which mediates downstream signaling to activate NF- κ B via multiple protein-protein interactions, protein modification, and signal-crosstalk (Fig.2, 3)³⁰.

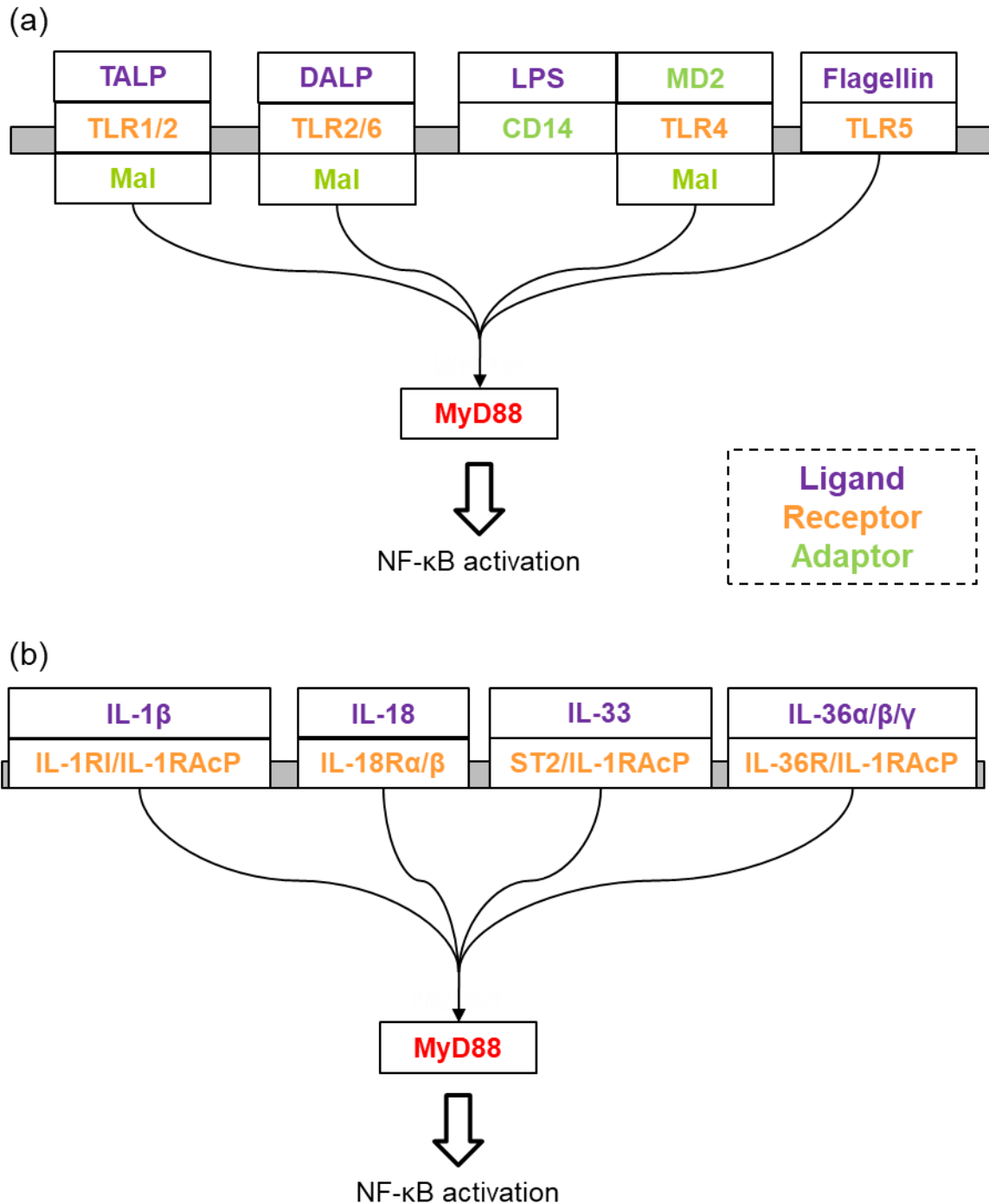


Figure 2 Schematic diagram of signal convergence at MyD88^{28,29}.

Signal transduction pathways of (a) TLRs at the plasma membrane and (b) IL-1fRs. MyD88 took the roll as a signal-convergence-hub, because these receptors commonly interact with MyD88 to induce NF-κB activation. TLR1/2, TLR2/6, TLR4, and TLR5 bind TALP (Triacyl lipoproteins), DALP (Diacyl lipoproteins), LPS, and fragelin, respectively. A part of TLRs indirectly interacts with MyD88 via an adaptor protein called Mal (MyD88-adapter-like). IL-1 family proteins, IL-1β, IL-18, IL-33, IL-36α/β/γ, activate MyD88 via corresponding receptors without Mal.

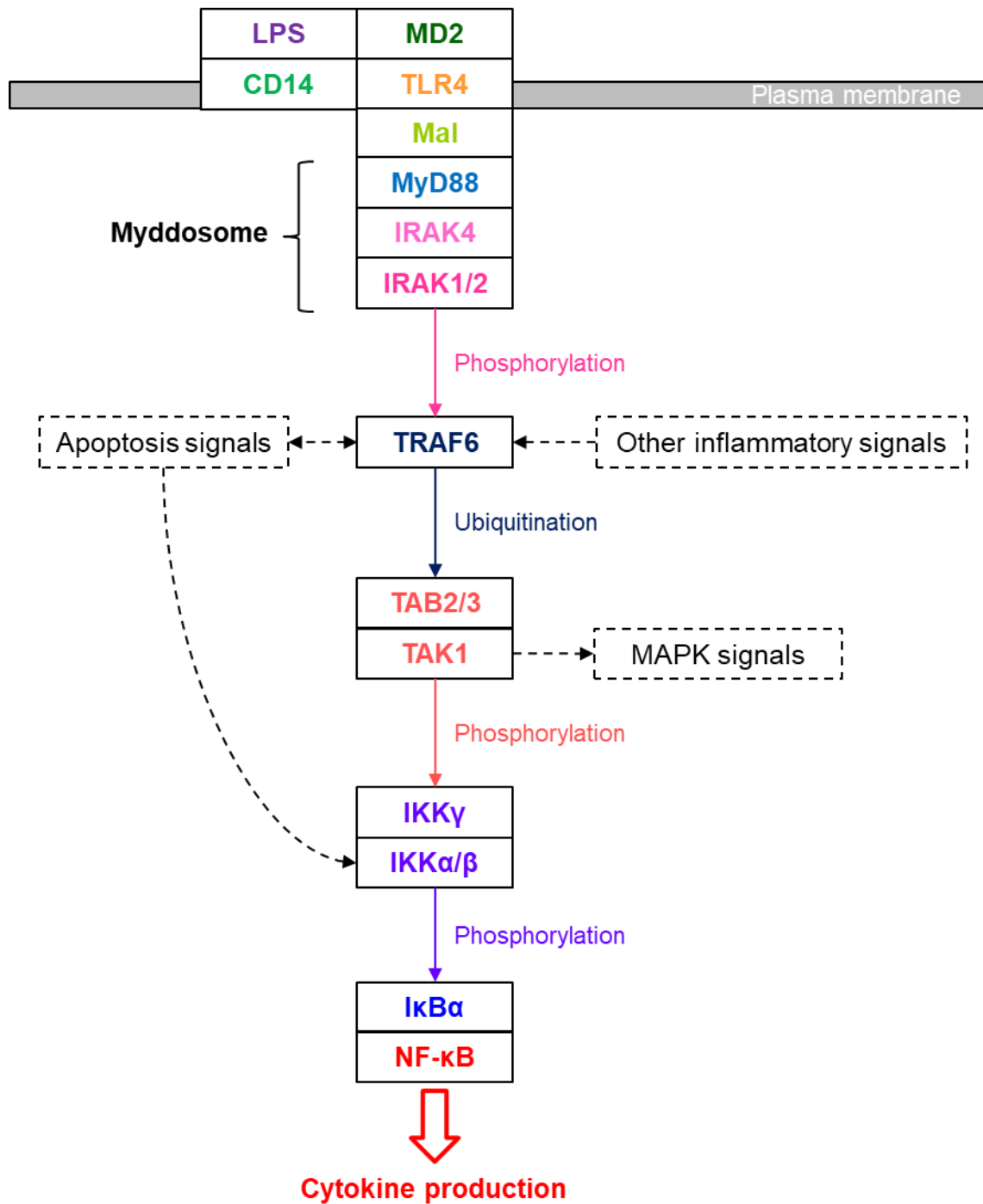


Figure 3 Schematic diagram representing the signal transduction pathway for TLR4-MyD88 dependent NF-κB signalling³⁰.

Multiple protein-protein interactions are shown tandem boxes. Protein modifications and signal-crosstalk are indicated as solid arrows and broken arrows, respectively.

6. MyD88

MyD88 functions as an adaptor between receptors and downstream kinases, leading to formation of a large complex called Myddosome³⁰. MyD88 comprises the N-terminal domain called DD (Death domain), the C-terminal domain called TIR (Toll/IL-1 receptors domain), and the linker region connecting them (Fig.4)^{31,32}. Two domains play core roles in forming the TLR-Myddosome complex via homotypic domain-domain interactions (Fig. 5).

TIR_{MyD88} (TIR of MyD88) is commonly used in most of the TLRs and IL-1fRs signaling (Fig.2), in which cytosolic TIR_{TLR} (TIR of TLRs) and IL-1fRs directly bind TIR_{MyD88}. However, some TLRs require an additional adaptor protein, Mal (MyD88-adaptor-like), which localized at plasma membrane to recruit MyD88 to the site³³. Interactions between TIR_{MyD88} and TIR of receptors activates DD_{MyD88} (DD of MyD88), and DD_{MyD88} assembles into a homo-tetramer via DD_{MyD88}-DD_{MyD88} homo-interaction. The homo-tetramer of DD_{MyD88} recruits kinases called IRAK4 (interleukin-1 receptor-associated kinase 4) and IRAK1/2 (interleukin-1 receptor-associated kinase 1 and 2) to form DD_{MyD88}-DD_{IRAK4}-DD_{IRAK1/2} hetero-oligomer via DD_{MyD88}-DD_{IRAK4} and DD_{IRAK4}-DD_{IRAK1/2} interactions (Fig.5)^{30,32}. This complex gathered by MyD88 is called Myddosome. The Myddosome formation activates kinase domain of IRAKs by auto-phosphorylation, which then phosphorylates TRAF6 (TNF receptor-associated factor 6). Phosphorylated TRAF6 induces cytokines production via the canonical NF-κB signal pathway (Fig.3).

Previous researches based on TIR-TIR interaction and DD-DD interaction have clarified the function of each domain of MyD88, however, how TIR_{TLR}-TIR_{MyD88} interaction leads to activation of DD_{MyD88} has not been uncovered. Although a previous report showed that there was no intermolecular interaction between DD_{MyD88} and TIR_{MyD88}, recent studies reported that DD_{MyD88} interacted with TIR_{MyD88}^{34,35}. If so, there is a possibility that the intramolecular interaction between DD_{MyD88} and TIR_{MyD88} regulates the activity of DD_{MyD88} that promotes formation of Myddosome.

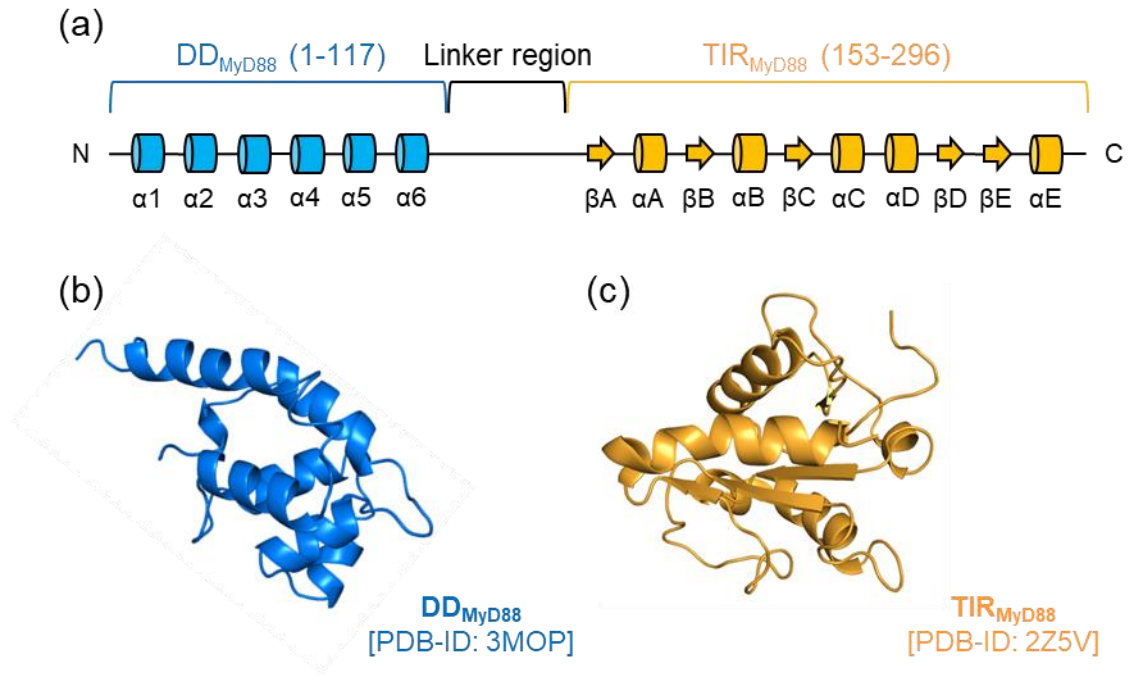


Figure 4 Molecular structures of MyD88.

(a) The secondary structure of $MyD88^{FL}$. (b, c) The molecular structure of DD_{MyD88} [PDB-ID: 3MOP]³² and TIR_{MyD88} [PDB-ID: 2Z5V]³¹.

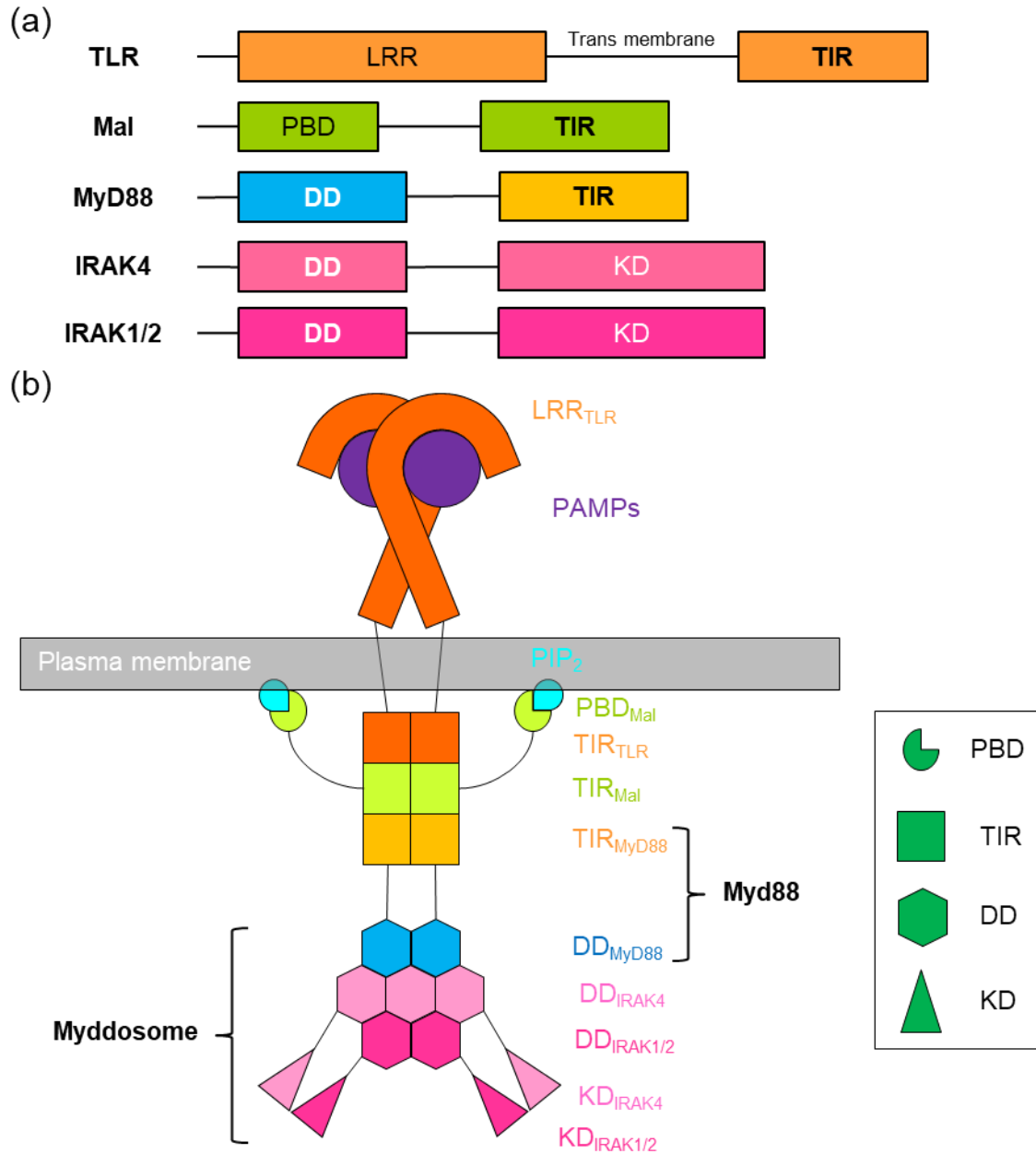


Figure 5 Schematic model of the TLR-Myddosome complex^{30,32}.

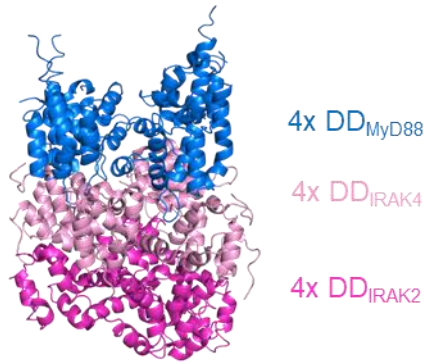
(a) Domain organizations of proteins comprised in the TLR-Myddosome complex. (b) A schematic model of the TLR-Myddosome complex. The molecular mechanism of the TLR-Myddosome complex functioned as active signal kinase complex is constituted from five steps protein-protein interactions, (1) the extracellular domain of TLR called LRR_{TLR} (leucine-rich repeat domain of TLR) binds to the corresponding PAMPs molecule, (2) this ligand binding induces dimerization of cytosolic TIR_{TLR}, (3) the TIR_{TLR} dimer recruits TIR_{Mal} (TIR of Mal) and TIR_{MyD88} via TIR-TIR interaction, (4) the TIR-complex assembly activates MyD88 and induces homo-oligomerization of DD_{MyD88}, and (5) the DD_{MyD88}-oligomer recruits DD_{IRAK4} and DD_{IRAK1/2} to form Myddosome. The Myddosome assembly makes KDs (kinase domains) close together that induces auto-activation of KDs by auto-phosphorylation. Activated KDs phosphorylate and activate TRAF6, which activates the NF- κ B canonical signal pathway. Incidentally, TIR_{Mal} usually localizes near membrane by the interaction between PIP₂ (Phosphatidylinositol 4,5-bisphosphate) and PBD_{Mal} (PIP2 binding domain of Mal).

7. Death domain superfamily

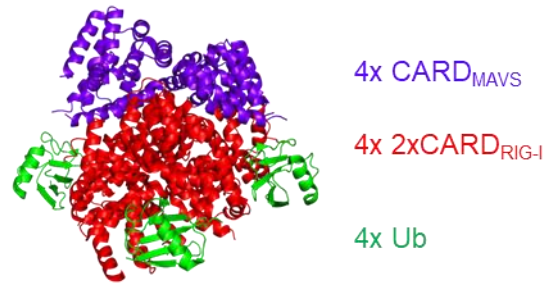
DDSF (death domain superfamily) is a group of proteins comprising death-fold domains characterized by six or seven tightly packed antiparallel α -helices^{36,37}. Death-fold domains are classified into DD (death domain), DED (death effector domain), CARD (caspase recruitment domain), and PYD (pyrin domain)³⁶. Death-fold domains have multiple homo-interaction sites and assemble into helical symmetric homo-oligomers by activation. Such homo-oligomerization of death-fold domains induce DDSF proteins to assemble into large oligomeric complexes called signalosomes, which mediate signals in apoptosis, inflammation, and pathogen recognition. Formation of signalosomes triggers the proximity-induced activation of enzymes involved in the signalosomes, contributing to amplification of the signals by simultaneously activating multiple enzymes^{38,39}. For instance, DD_{MyD88} forms Myddosome with IRAKs, whereby IRAKs are activated by auto-phosphorylation (Fig.5)^{30,32}.

Signalosomes comprise two modules; core oligomeric module and circumferential functional module. The core oligomeric modules adopt multi-layer structures constituted from helical oligomers of death-fold domains (Fig.6a-d)^{32,40–42}. In the structure, the primal layer is constituted from 4-5 death-fold domains via homo-interactions, to which next layer of other death-fold domains bind. For example, the layer of DD_{MyD88} tetramer recruits DD_{IRAK4}³² to form the second layer. Intriguingly, interactions between monomeric DD_{MyD88} and DD_{IRAK4} are very weak, whereas tetramerization strengthens the affinity of the interactions. On the other hand, some DDSF proteins oligomerize in a totally different manner, as they do not require death-fold domains to do so (Fig.6e, f)^{43,44}.

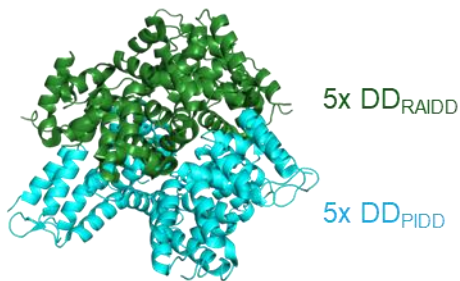
(a) Myddosome



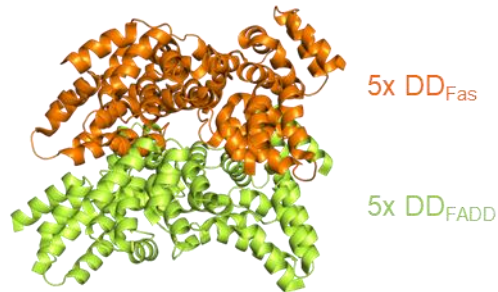
(b) MAVS-Signalosome



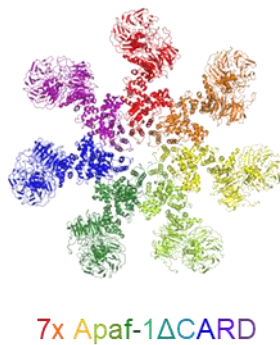
(c) PIDDosome



(d) DISC



(e) Apoptosome



(f) NLRC4-inflammasome

**Figure 6 Molecular structures of DDSF-signalosomes.**

A DDSF signalosome comprises two modules, a core oligomeric module, and a circumferential functional module. Molecular structures of the core oligomeric modules of, (a) Myddosome [PDB-ID: 3MOP]³², (b) MAVS-Signalosome [PDB-ID: 4P4H]⁴⁰, (c) PIDDosome [PDB-ID: 2OF5]⁴¹, and (d) DISC [PDB-ID: 3OQ9]⁴², were revealed as heterotypic death-fold domain complexes with helical symmetries. Molecular structures of the circumferential functional modules of, (e) Apoptosome [PDB-ID: 5WVE]⁴³ and (f) NLRC4-Inflammasome [PDB-ID: 3JBL]⁴⁴, were revealed as circular symmetric complexes without death-fold domains.

There are some DDSF proteins comprising multiple death-fold domains, such as ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain)³⁶. ASC is an adaptor protein linking between caspases and some PRRs, such as AIM2 and NLRP3, and it comprises PYD_{ASC} (PYD of ASC) and CARD_{ASC} (CARD of ASC)³⁹. Ligand binding of AIM2 induces assembly of its PYD, to which multiple PYD_{ASC} are recruited to elongate the PYD filament. Then, multiple pro-caspase-8 DED are recruited and the PYD/DED filament further extends (Fig.7)³⁸. Thus, PYD_{ASC} functions as a core oligomeric module.

Moreover, the PYD/DED filament brings multiple CARD_{ASC} into a close proximity, which provides bases for elongating the CARD filament. Pro-caspase-1 are then recruited for elongating the CARD filament. In this case, CARD_{ASC} works as a core oligomeric module (Fig.7)³⁸. This complicated multi-oligomer assembly showed that a protein comprising multiple oligomeric domains assembles into a multi-axis complex comprising multiple filaments.

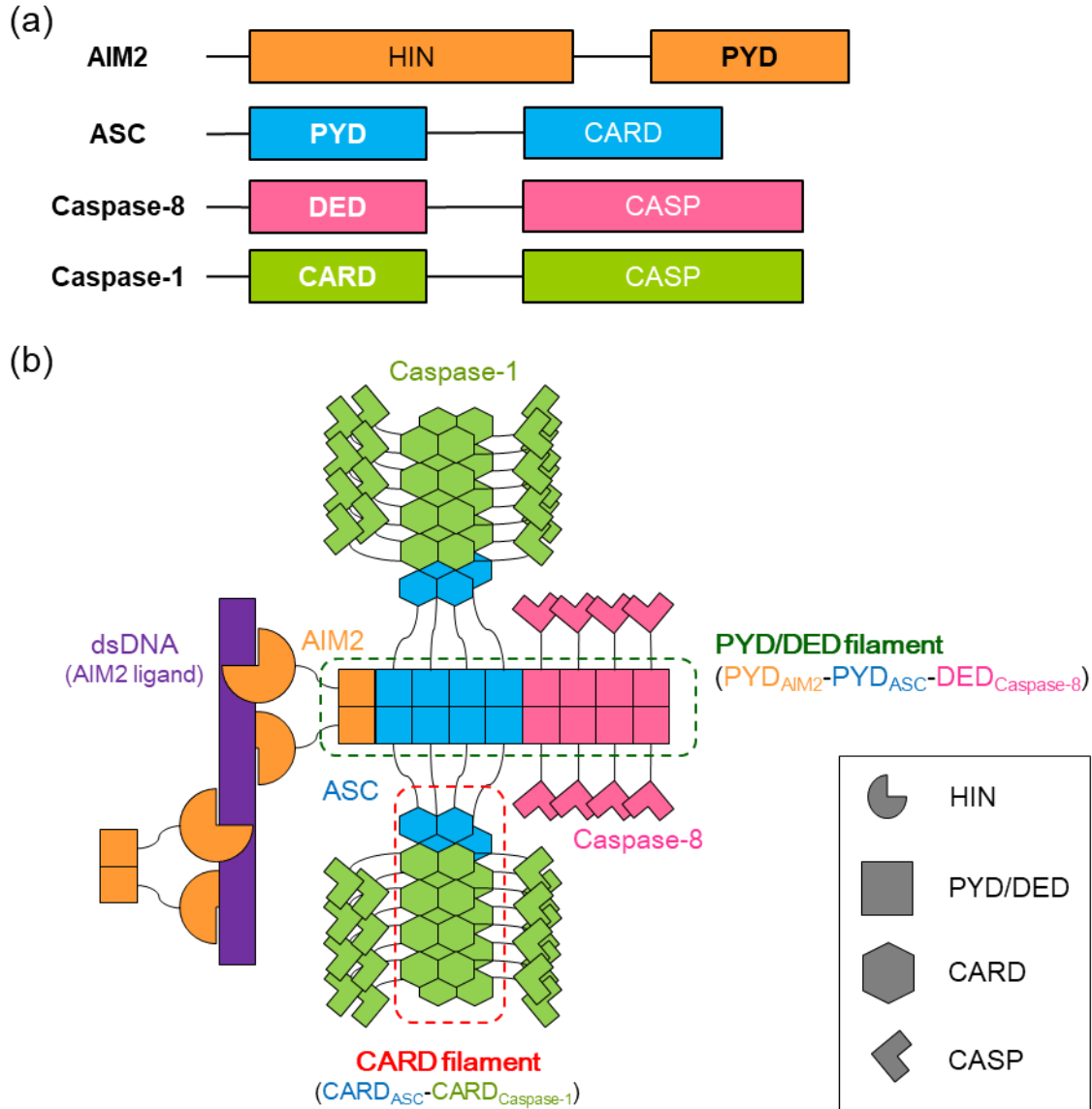


Figure 7 Model for multi-axis filament assembly with ASC³⁸.

(a) Domain organizations of proteins comprised in AIM2-inflammasome. HIN (HINa-like modes of DNA binding), PYD (pyrin domain), DED (death effector domain), CARD (caspase recruitment domain), and CASP (caspase protease domain) contribute to ligand recognition, formation of the PYD filament, formation of the CARD filament, and sequence-specific cleavage of peptide bonds, respectively. (b) A schematic structure model of AIM2-inflammasome. The AIM2-inflammasome initiation complex (dsDNA-AIM2) nucleates and triggers elongation of an ASC PYD filament. Clustered CARD_{ASC} on the side of the ASC filament recruit procaspase-1 and nucleate to trigger elongation of procaspase-1 CARD filaments. Catalytic domains on the sides of the pro-caspase-1 CARD filament can dimerize and become activated. Pro-caspase-8 DEDs are recruited to the ASC PYD filament, and a PYD/DED filament is nucleated. The pro-caspase-8 catalytic domains on the side of the filament can also dimerize and become activated.

8. TIR superfamily

TIRSF (Toll/IL-1 receptor superfamily) comprises proteins that contain the phylogenetically conserved TIR (Toll/IL-1 receptor domain)⁴⁵. TIRSF proteins in vertebrates are classified into three groups; TLRs, IL-1fRs, and cytosolic adaptor proteins. The cytosolic adaptor proteins are MyD88, Mal, TRIF (TIR domain-containing adaptor molecule-inducing interferon- β), TRAM (TRIF-related adaptor molecule), SARM (Sterile α and armadillo motif-containing protein), and BCAP (B-cell adaptor for phosphoinositide 3-kinase)^{31,45–49}. All these adaptor proteins function in TLRs signaling, but each member has a different function. As aforementioned, MyD88 functions as a common signal transduction adaptor for IL-1fRs and all TLRs except TLR3, and Mal mediates interactions between MyD88 and a part of TLRs such as TLR1/6, TLR2/6, and TLR4. TRIF and TRAM play a similar role to MyD88 and Mal as the signal transduction adaptor for TLR3 and endosomal TLR4, respectively. SARM inhibits signal transductions mediated by MyD88 and TRIF⁴⁸. BCAP inhibits the membrane localization of Mal⁴⁹.

Although not all molecular structures of TIR were revealed, several structures had been determined (Fig.8)^{31,45–47,49–54}. TIRs fold into a common motif comprising a five-stranded core parallel β -sheet surrounded by multiple outer α -helices⁵⁵. TIRs have a common tendency to form homo-dimer and to interact with other TIR, but it had not been thought that TIRs form large oligomers like DDSF-signalosomes^{45,56}. However, a recent report showed that TIR_{Mal} assembled into a fibril structure, and its structure was revealed by cryo-EM analysis at atomic level⁵⁷. In the report, TIR_{MyD88} was also shown to assemble into fibrils when seeded with a small amount of TIR_{Mal}. Interestingly, TIR_{MyD88} did not form any fibrils on its own. These observations suggest that by assembling into fibrous oligomers TIRs play similar roles as DDSF-signalosomes play for signal transductions.

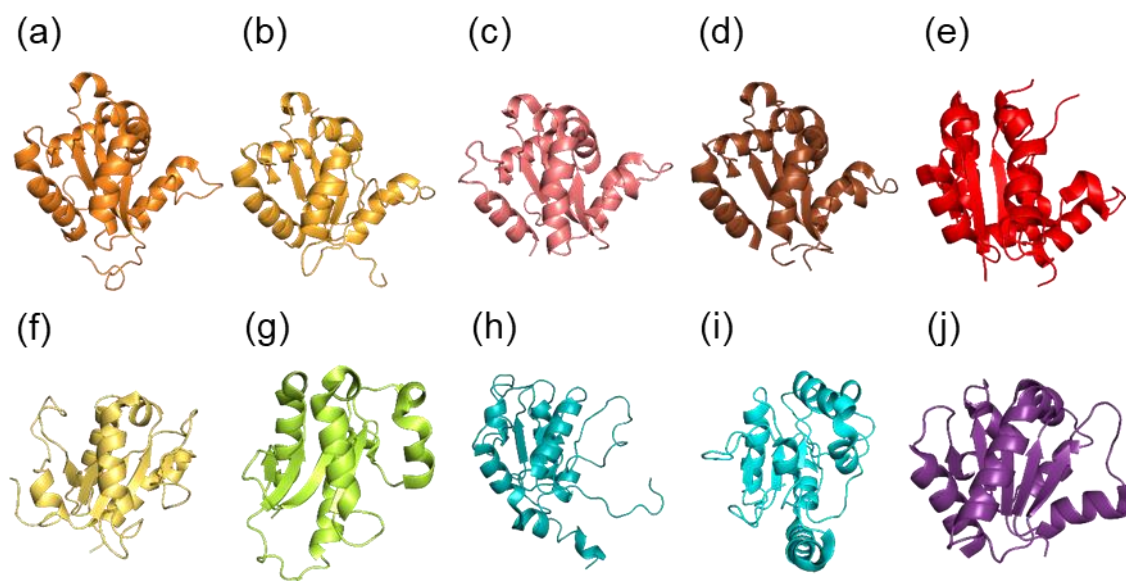


Figure 8 Molecular structures of monomeric TIRs.

Molecular structures of TIR of (a) TLR1 [PDB-ID: 1FYV]⁵¹, (b) TLR2 [PDB-ID: 1FYW]⁵¹, (c) TLR6 [PDB-ID: 4OM7]⁵³, (d) TLR10 [PDB-ID: 2J67]⁵⁴, (e) IL-1RAcP (IL-1R accessory protein-like 1) [PDB-ID: 1T3G]⁵², (f) MyD88 [PDB-ID: 2Z5V]³¹, (g) Mal [PDB-ID: 3UB2]⁴⁶, (h) TRAF [PDB-ID: 2M1X]⁴⁷, (i) TRIF [PDB-ID: 2M1W]⁴⁷, and (j) BCAP [PDB-ID: 5FOR]⁴⁹ show that they fold into a common motif; core β -strands surrounded by α -helices.

9. Diseases related to MyD88

Since MyD88 widely contributes to early immune responses, genetic disorders of MyD88 can affect the whole immune system. Deficiency of MyD88 and some mutations in DD_{MyD88} both cause immunodeficiency^{58–60}. Considering the positions of disease related mutations in the molecular structure of DD_{MyD88}–oligomer, which are in the DD-DD interface, and a similarity in symptom between the MyD88 deficiency and the DD_{MyD88} mutations, it was thought that these genetic disorders spoil DD_{MyD88}–DD_{MyD88} or DD_{MyD88}–DD_{IRAK4} interaction^{32,59}. If this happens, the downstream signaling will be shut off and immune responses will not occur, leading to the immunodeficiency.

On the other hand, tumor genetic mutation analyses of liquid biopsies have identified gain-of-function mutations of TIR_{MyD88}, some of which are frequently found in lymphomas such as ABC (activated B-cell-like) subtype of DLBCL (diffuse large B-cell lymphoma)^{61,62}, in which NF-κB and JAK-STAT (Janus kinase-signal transducers and activators of transcription) signaling were revealed to be constitutively activated, thus contributing to survival of malignant cells. One representative of such mutations is L265P (also annotated as L252P)^{59,63}. The mutated residue is located in the hydrophobic core of TIR_{MyD88}; nevertheless, three studies based on molecular simulations proposed that the L252P mutation would not destroy the tertiary structure of the TIR domain^{64–66}. A report suggested that an altered dynamic character of the TIR domain caused by the mutation may promote the spontaneous oligomerization of TIR_{MyD88} (L252P)⁶⁵, which can cause the oligomerization of DDs and thus Myddosome formation. Therefore, receptor activation is not necessary to trigger signaling, consistent with the constitutive activation observed in the reported lymphomas^{61,63,67}. However, details of core molecular mechanism that induce the constitutive activation are still unclear.

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Chapter II

Auto-suppression mechanism of full-length MyD88 based on intramolecular interaction

Abstract

MyD88 (myeloid differentiation factor 88) is an important protein in innate immunity. Two structural domains of MyD88 have been well characterized separately, but the global architecture of full-length MyD88 remained unclear. Here, we propose an autosuppressive mechanism of MyD88 regulated by the intramolecular interaction between the two domains.

1. Introduction

MyD88 is an adaptor protein that functions in the innate immune response and inflammatory signaling¹, which are activated by TLR (Toll-like receptor) and IL-1R (interleukin-1 receptor) family members. MyD88 comprises two protein-protein interaction modules: DD_{MyD88} (death domain) at the N-terminus and TIR_{MyD88} (Toll/IL-1R domain) at the C-terminus. These domains are connected by an ~35 amino acid residue linker (Fig. 1)^{2,3}. The primary function of MyD88 has been well established: MyD88 bridges membrane receptors (TLRs and IL-1Rs) and cytosolic kinases called IRAKs (interleukin-1 receptor-associated kinases), in which the homo- and heterotypic interactions of both TIRs and DDs play critical roles. For instance, in TLR4 signaling, a component of pathogenic bacteria, lipopolysaccharide, binds the extracellular domain of TLR4 with cofactors, inducing homodimerization of the extracellular regions of TLR4 and bringing the cytosolic regions of two TLR4 molecules into close proximity⁴, enabling TIR_{TLR4} (TIR domain of TLR4) to dimerize underneath the membrane. The dimerized TIR_{TLR4} provides a combined surface that recruits Mal/TIRAP (MyD88 adapter-like/TIR-containing cytosolic adaptor proteins) and MyD88 via heteromeric oligomerization of the TIR domains of these proteins. Upon the formation of this complex containing the activated receptors, MyD88 starts to further recruit IRAK4 and IRAK1/2 to form a large protein complex termed the Myddosome, which is mediated by oligomeric interactions between the DDs of MyD88, IRAK4 and IRAK1/2. Myddosome formation results in the activation of IRAK4, which triggers downstream signaling to activate NF- κ B and AP-1 to produce inflammatory cytokines.

The crystal structure of DD_{MyD88} in a complex with DD_{IRAK4} and DD_{IRAK2} was solved³, providing a clear picture of how DD_{MyD88}, DD_{IRAK4} and DD_{IRAK2} form the complex. However, although the structures of isolated TIRs of MyD88 and other TIR-containing adaptor proteins have been reported⁵⁻⁸, the details of how they form dimers/oligomers have not been well understood. Numerous biochemical studies of TIR_{MyD88} were conducted to identify sites on its surface that are critical for dimeric/oligomeric association, based on proposed structural models⁹. While DD_{MyD88} and TIR_{MyD88} have each been well characterized, their relative positions in a full-length context, namely, the global architecture of MyD88, have never been explored. In this study, to gain insights into the regulation mechanism of MyD88, which might be governed by its global architecture,

we investigated the interdomain interaction of MyD88 by using NMR spectroscopy and HS-AFM (high speed atomic force microscopy).

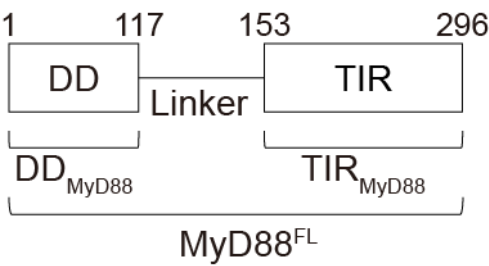


Figure 1 The domain organization of MyD88.

2. Material and Method

2.1 Construction of expression vectors

The coding region of MyD88^{FL} (MyD88, NM_002468.4, residues 14-309, annotated as 1-296 in this study) was amplified from the human cDNA library by polymerase chain reaction and cloned into a pET-28a vector [Novagen, WI, USA], which was engineered with a 10×His-tagged N-terminal SUMO-3 (small ubiquitin-like modifier-3) (NM_006936.2, residues 14-92) for protein expression and purification. The plasmids for DD_{MyD88} (residues 1-152) and TIR_{MyD88} (residues 153-296) were derived from the MyD88^{FL} construct. The plasmid of DD_{MyD88} was cloned into a pGEX-6P-1 vector [GE Healthcare, IL, USA], and the plasmid of TIR_{MyD88} was cloned into a pET-28a vector, which was engineered with a 6×His-tagged GB1 (Streptococcal protein GB1 domain, residues 261-324). The constructs for the derivatives, such as the alanine-substituted derivatives, were prepared by site-directed mutagenesis.

2.2 Protein expression and purification

For experimental samples, the expression vector for either the His-GB1 tag fusion TIR_{MyD88}, GST tag fusion DD_{MyD88} or His-SUMO tag fusion MyD88^{FL} was transformed into *E. coli* BL21(DE3) cells. For isotope labeling, the cells transformed with the His-GB1 tag fusion TIR_{MyD88} vector were grown in either M9 minimal medium containing ¹⁵NH₄Cl or a premixed medium, *E.coli* OD1 DN [Silantes GmbH, Bayern, Germany]. The cells transformed with the GST tag fusion DD_{MyD88} vector or His-SUMO tag fusion MyD88^{FL} vector were grown in LB medium. The His-GB1 tag fusion TIR_{MyD88} and GST tag fusion DD_{MyD88} were first purified with affinity column chromatography. After removal of each tag with HRV3C (human rhinovirus 3C protease), the protein was further purified with ion exchange chromatography and size-exclusion chromatography.

The His-SUMO tag fusion MyD88^{FL} was expressed as inclusion bodies. The washed cell debris was resuspended with denaturation buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM NaCl and 6 M guanidine chloride. The denaturated His-SUMO tag fusion MyD88^{FL} was purified with affinity column chromatography. Eluted His-SUMO tag fusion MyD88^{FL} was refolded by the rapid dilution method with ten times volume dilution buffer containing 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 400 mM arginine chloride and 20 mM

dithiothreitol. After removal of the His-SUMO tag with SENP2 (sentrin-specific protease 2), refolded MyD88^{FL} was further purified with size-exclusion chromatography.

2.3 NMR spectroscopy

NMR spectra were measured by Bruker Avance II 700 MHz and Avance II 800 MHz spectrometers equipped with cryogenic probes [Bruker, MA, USA] at 298 K. The samples for NMR analyses were dissolved in NMR buffer containing 20 mM potassium phosphate (pH 6.0), 150 mM NaCl, and 10 mM DTT with the addition of 5 v/v % D₂O. To detect the interaction between TIR and DD, 10 μ L of 300 μ M unlabeled DD or its derivatives in NMR buffer was added to 240 μ L of 50 μ M [¹H, ¹⁵N]-TIR.

For TCS (transferred cross-saturation) experiments, a sample containing 65 μ M [²H, ¹⁵N]-TIR and 6.5 μ M DD was dialyzed by RE buffer containing 20 mM potassium phosphate (pH 6.0), 150 mM NaCl, 50 mM Arg-HCl, 50 mM Glu-NaOH, 10 mM DTT and 80 v/v% D₂O. The TCS experiments was conducted in accordance with a previously reported procedure¹⁰. The maximum RF amplitude was 0.17 kHz for WURST-2 (adiabatic factor Q0 = 1). The saturation frequency was set at 0.9 p.p.m. The measurement times were 65 h with a recycle delay of 4.5 s [3.0 s for the adjusting delay and 1.5 s for the saturation time]. All spectra were processed via NMRPipe¹¹ or Bruker TopSpin 3.5 [Bruker, MA, USA] and analyzed using Sparky¹².

2.4 High-speed AFM imaging

A HS-AFM (high-speed atomic force microscope) was equipped with a small cantilever (BL-AC10-DS-A2 [Olympus, Tokyo, Japan]: spring constant, k = 0.1 N/m, resonance frequency, f = 400~500 kHz in water) and was operated in tapping mode at room temperature^{13–15}. An amorphous carbon tip was grown on the top of each cantilever by electron-beam deposition with a scanning electron microscope (ERA-8000FE [Elionix, Tokyo, Japan]). The free oscillation amplitude was 1.4~2 nm, and the typical set-point amplitude was 85% of the free oscillation amplitude. A mica disk (1 mm in diameter) fixed by epoxy glue on a glass rod (2 mm in diameter and 2 mm in height) was used as a sample stage¹⁴. Before or after the sample stage was fixed by nail polish on the z-piezo of the HS-AFM scanner, the mica was freshly cleaved. Freshly cleaved mica and

AP-mica (3-aminopropyl-trietoxy silane modified mica) were used as substrates. AP-mica was prepared by depositing 0.05% 3-aminopropyltriethoxysilane [Shin-Etsu Chemical, Tokyo, Japan] on freshly cleaved mica and left for 3 min¹⁶. Then, 5 μ L of 10 nM MyD88^{FL} (WT) or its derivatives was adsorbed on the substrates in AFM sample buffer containing 20 mM MES-NaOH (pH 6.0) and 10 mM DTT. After 5 min, the sample was rinsed with 20 μ L of sample buffer.

2.5 Processing of HS-AFM data

The HS-AFM image sequences were processed using ImageJ software ver. 1.50i¹⁷, additional plugins for ImageJ and Kodic 4.5.7.25 developed by the Kanazawa-Univ Bio-AFM Frontier Research Center¹⁸. All images applied polynomial fitting in both the x- and y-direction and median filter in 1.0 pixel, and adjusted the background height level to zero after background leveling¹⁹.

D_{cc} was measured and analyzed by the ImageJ plugin, MTrackJ²⁰. The collected data were processed with Excel 2010 [Microsoft, WA, USA] and R²¹.

3. Result

3.1 NMR titration to determine intermolecular interaction interface on DD_{MyD88}

We first recorded the NMR spectra of isolated ¹⁵N-labeled TIR_{MyD88} in the absence or presence of DD_{MyD88} (Fig. 2a). The amide ¹H-¹⁵N NMR signals of ¹⁵N-TIR_{MyD88} in its free form were dispersedly distributed with substantial intensities, but many of these signals started to decrease when DD_{MyD88} was added (Fig. 2b). The reduction in NMR signals depended on the amount of added DD_{MyD88}, and the signals mostly disappeared when two eq (molecular equivalents) of DD_{MyD88} was added. Similar to other DD superfamily members²², DD_{MyD88} was previously demonstrated to form self-oligomers^{23,24}, which underlie the signaling DD_{MyD88}-DD_{IRAK4}-DD_{IRAK2} complex, the Myddosome³. We thus interpreted the reduction in NMR signals as indicating that ¹⁵N-TIR_{MyD88} bound such functionally relevant DD_{MyD88} oligomers but not resulting from non-specific aggregations (see Fig. 3). Once ¹⁵N-TIR_{MyD88} binds to such oligomers, the NMR signals would broaden and weaken due to the shorter transverse relaxation time resulting from the slower molecular tumbling rate of the large molecular assembly.

After the direct interaction between DD_{MyD88} and ¹⁵N-TIR_{MyD88} was revealed, we performed the same NMR titration experiment using DD_{MyD88} derivatives harboring amino acid substitutions. Two single amino acid-substituted derivatives, DD_{MyD88} (E52A) and DD_{MyD88} (Y58A), and a triply substituted derivative (E52Q-E53Q-D55N) named DD_{MyD88} (EQ) were tested (Fig. 2d). These derivatives did not show any decrease in the NMR signals of ¹⁵N-TIR_{MyD88} in the presence of DD_{MyD88} (Fig. 2b), indicating that the substituted residues (E52, E53, D55, and Y58) critically mediate the DD_{MyD88}-TIR_{MyD88} interaction (Fig. 2c). A previous mutational study of MyD88 found that E52 and Y58 were important in recruiting IRAK4 (Fig. 4a)²⁵. Additionally, three anionic residues, E52, E53 and D55, were reported to play roles in mediating the interaction between DD_{MyD88} and DD_{IRAK4} (Fig. 4b)³. Thus, it is possible that TIR_{MyD88} interferes with the signaling-related DD_{MyD88}-DD_{IRAK4} interaction by masking the interface on DD_{MyD88}, suggesting the possibility of an autosuppression mechanism of MyD88.

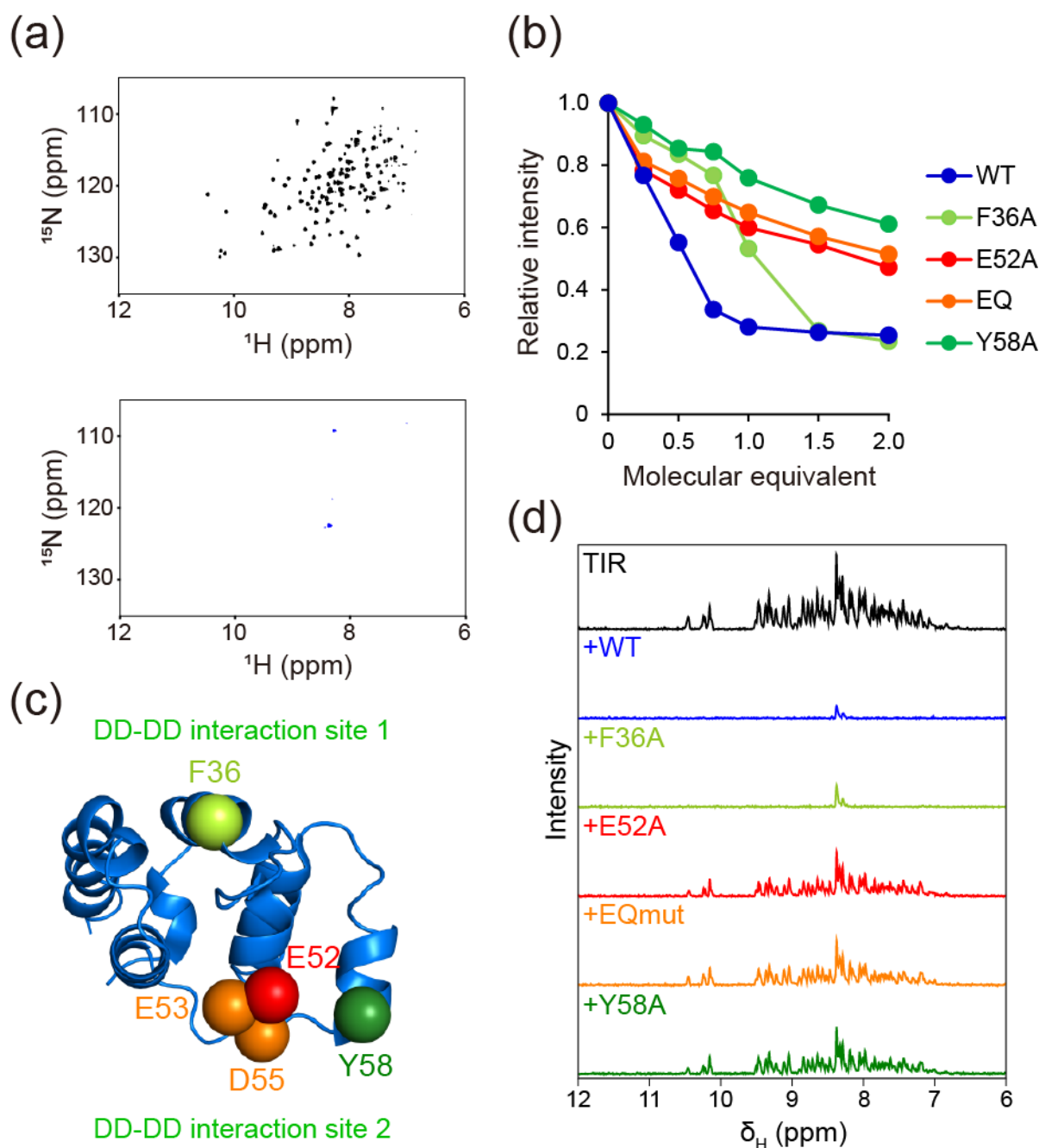


Figure 2 NMR titration experiments to identify the binding sites of DD_{MyD88} for ^{15}N -TIR_{MyD88}.

(a) ^1H - ^{15}N SOFAST-HMQC spectra of ^{15}N -TIR_{MyD88} without (top) and with (bottom) 2 eq. of DD_{MyD88}. (b) Plot of the relative intensities of the NMR signals of ^{15}N -TIR_{MyD88} titrated with DD_{MyD88} variants. The relative intensity was calculated as the ratio of the integrated value of the peak intensity from 6.0 ppm to 12.0 ppm at each titration point relative to that of the DD_{MyD88} free state in the ^1H 1D projected spectra. (c) Position of the replaced amino acids (C α) shown on the structure of DD_{MyD88} (PDB ID: 3MOP). (d) ^1H 1D projections of the ^1H - ^{15}N SOFAST-HMQC spectra of ^{15}N -TIR_{MyD88} in the absence and presence of 2 eq of DD_{MyD88} derivatives.

The attenuation of WT (blue) and F36A (yellow-green) was saturated before the addition of two molecular equivalents DD_{MyD88}, while that of E52A (red), EQ (E52Q-E53Q-D55N) (orange) and Y58A (green) was not. The weak attenuation suggests that these mutations (E52A, EQ and Y58A) inhibited the interaction between DD_{MyD88} and ^{15}N -TIR_{MyD88}.

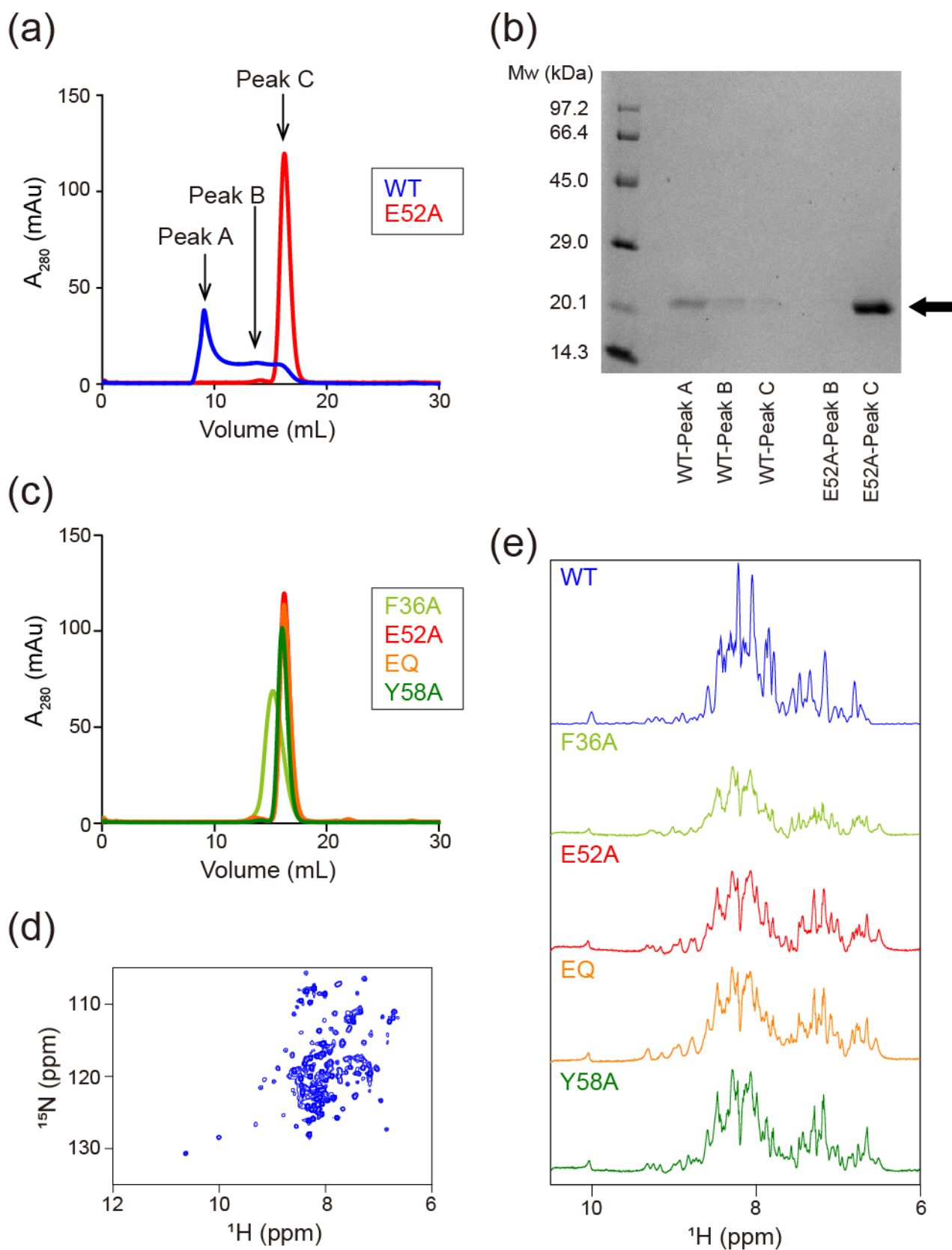


Figure 3 The global characters of DD_{MyD88} (WT) and mutants.

(a) Size-exclusion chromatograms of DD_{MyD88} (WT) (blue) and DD_{MyD88} (E52A) (red). The extremely broadened peak of DD_{MyD88} (WT) suggests the formation of complexes of various molecular weights, while the introduction of the E52A mutation led to dissociation of the complexes. The estimated size of DD_{MyD88} (E52A) from the

profile roughly corresponds to a dimer. (b) SDS-PAGE showed that DD_{MyD88} (WT) was present in most of the fractions. (c) Size-exclusion chromatograms of DD_{MyD88} mutants (F36A; yellow green, E52A; red, EQ; orange and Y58A; green). The analysis was performed under the same conditions as in (a). The chromatograms suggest that all DD_{MyD88} mutations led to the dissociation of the various molecular weight complexes (2-4-mers). (d) ¹H-¹⁵N SOFAST-HMQC spectra of ¹⁵N-DD_{MyD88} (WT) measured in the same buffer conditions as in Fig. 2a. (e) Comparison between the ¹H 1D projected spectra of (d) and the ¹H 1D spectra of DD_{MyD88} mutants. The lack of a marked difference between the spectra of the WT and mutants suggests that all mutants formed a similar conformation to the WT.

DD_{MyD88} was previously demonstrated to form self-associated oligomers in solution in a reversible manner^{23,24}. Although structures of oligomers exclusively formed by DD_{MyD88} have not been determined, the crystal structure of the complex comprising DD_{MyD88}, DD_{IRAK4} and DD_{IRAK2} provided critical insights into the self-oligomers of DD_{MyD88}³, in which six copies of DD_{MyD88} assembled continuously to form a helical ring. Although the tetramer-like side of the ring was bound by DD_{IRAK4} (Fig. 4b), the hexamer was largely held together via self-associative interactions between multiple DD_{MyD88}. According to this observation, DD_{MyD88} should have a propensity to form self-associated oligomers in solution. The concentration of DD_{MyD88} in our NMR titration experiments was 10-100 μ M. At this concentration, DD_{MyD88} begins to form self-oligomers as DD_{MyD88} was shown to exist as monomers at concentrations less than 0.4 mg/ml ($\sim$ 27 μ M) but to begin to form self-oligomers at higher concentrations²³. Consistent with this report, we also observed the emergence of larger oligomers of DD_{MyD88} at 100 μ M (Fig. 3a). Thus in our NMR titration, DD_{MyD88} would have existed as such self-oligomers. Consistently, all the mutated DD_{MyD88} we created, which had mutations at the homotypic DD-DD interface of the DD_{MyD88} hexamer in the crystal structure, showed substantial decreases in self-associative nature and existed as 2-4-mers (Fig. 3a-c), indicating that the DD_{MyD88} oligomer we observed in solution is closely related to that in the crystal.

Widespread disappearance of NMR signals by itself cannot discriminate functionally relevant interactions from nonspecific aggregations. Nevertheless, from the words above, we interpreted the disappearance of NMR signals in our titration experiment to be due to the interaction between ¹⁵N-TIR_{MyD88} and functionally relevant oligomeric states of DD_{MyD88}, which thus reflects the functional significance of MyD88.

In contrast to these derivatives, DD_{MyD88} (F36A) caused a decrease in the NMR signals, as wild-type DD_{MyD88} (WT) did (Fig.2b), demonstrating that F36 is not involved in the DD_{MyD88}-TIR_{MyD88} interaction. In the crystal structure of the Myddosome assembly (complex of DD_{MyD88}-DD_{IRAK4}-DD_{IRAK2}), F36 is deeply involved in the DD_{MyD88}-DD_{IRAK4} interface (Fig. 4a); thus, this residue is likely involved in the DD_{MyD88}-DD_{IRAK4} interaction but not in the intramolecular DD_{MyD88}-TIR_{MyD88} interaction.

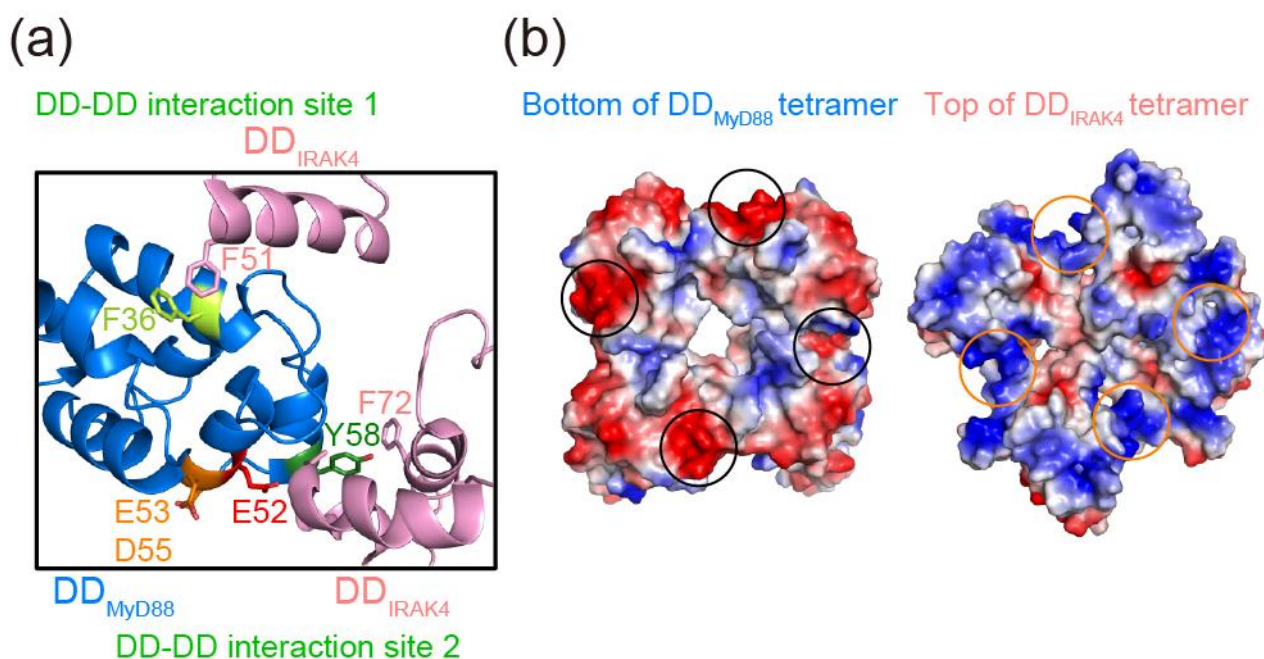


Figure 4 Interaction sites between DD_{MyD88} and DD_{IRAK4}.

(a) Interaction sites between DD_{MyD88} and DD_{IRAK4} in the structure of the DD oligomer (PDB ID: 3MOP)³. (b) Charge distribution of interaction surfaces between the DD_{MyD88} tetramer and the DD_{IRAK4} tetramer (PDB ID: 3MOP). Anionic sites formed by E52, E53 and D55 indicated by black circles on the DD_{MyD88} tetramer played important roles in interacting with cationic sites on DD_{IRAK4} (orange circles).

3.2 Transfer cross-saturation NMR to determine intermolecular interaction interface on TIR_{MyD88}

Having demonstrated the interaction between TIR_{MyD88} and DD_{MyD88} and identified critical amino acid residues on DD_{MyD88}, we next explored the TIR_{MyD88} surface for the sites responsible for binding DD_{MyD88}. To this end, a TCS-NMR (transfer cross-saturation NMR) experiment was conducted on the [²H, ¹⁵N]-TIR_{MyD88} sample²⁶. In TCS-NMR, amino acid residues whose amide ¹H showed significantly decreased NMR signals were considered to have experienced saturation transfer at the molecular interface. As shown in Fig. 5a, the NMR signals of [²H, ¹⁵N]-TIR_{MyD88} displayed 20-30% or greater attenuation in several regions, highlighting the residues that form contacts at the interface with DD_{MyD88}. These significantly attenuated residues (>30%), D162, V193, R218, M219, K256 and L293, illustrated as green spheres in Fig. 5b, were mostly found in the inside of the positively charged region, as mapped on the TIR structure in Fig. 5b, which comprises basic amino acid residues: R217, R218, K250 and R251. This finding is consistent with the negative charges of the interacting residues found in DD_{MyD88}. Therefore, electrostatic interactions likely dominate the TIR_{MyD88}-DD_{MyD88} interface.

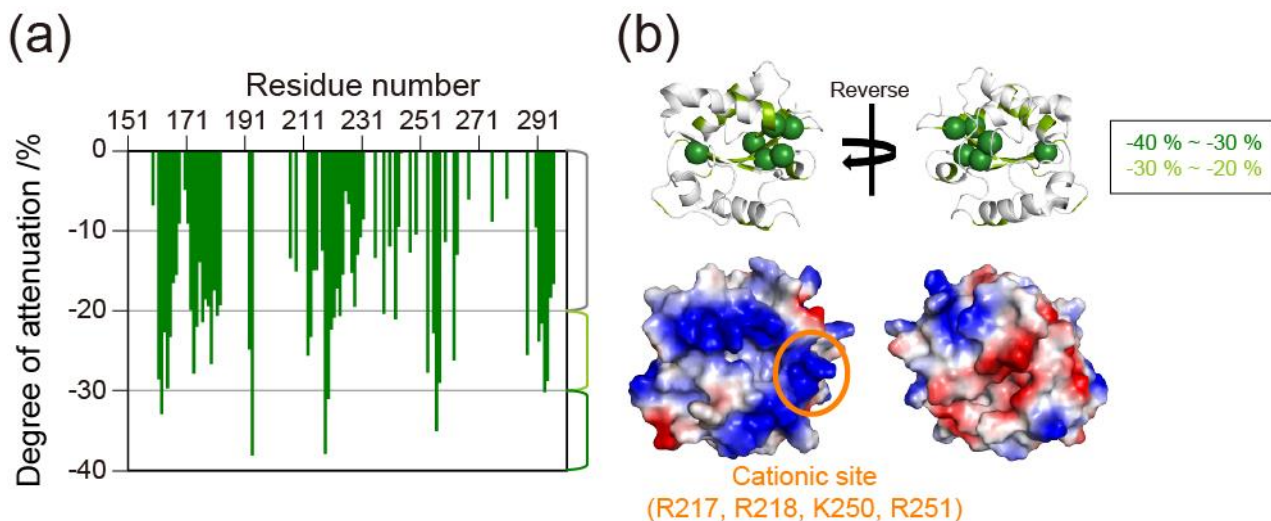


Figure 5 TCS-NMR experiments to identify DD_{MyD88} interaction sites on [²H, ¹⁵N]-TIR_{MyD88}.

(a) The degree of NMR signal attenuation of [²H, ¹⁵N]-TIR_{MyD88} caused by TCS plotted as a function of amino acid residue number. Gray columns indicate no data available. (b) The residues whose amide ¹H NMR signals substantially decreased are shown (Ca) in the ribbon model of TIR_{MyD88} (PDB ID: 2Z5V), in which residues attenuated >30%, 20-30% and <20% and no data are illustrated as green spheres, yellow-green, white and gray, respectively. The surface electrostatic potential of the molecule is also shown. Blue and red indicate positive and negative potentials, respectively.

3.3 High-speed AFM measurement to detect intermolecular interaction in MyD88^{FL}

Although we have demonstrated the interactions between the separated TIR_{MyD88} and DD_{MyD88} by using NMR, in principle, those are intermolecular interactions. To experimentally verify the native intramolecular DD_{MyD88}-TIR_{MyD88} interaction, analysis of MyD88^{FL} at the single molecule level is ideal. We thus employed HS-AFM, with which dynamic motions of single molecules can be visualized with nanometer spatial resolution and time resolution in the tens of milliseconds. Importantly, the technique can be applied to samples in aqueous media at room temperature and under ordinary pressure. Hence, the dynamics of global conformational changes under near-native conditions can be visualized, although the sample proteins must be weakly bound to the AFM stage. This association restricts the translational motion of the sample proteins to some extent. Nevertheless, HS-AFM would be sufficient to judge whether two domains are apart or in close proximity to make direct contact.

We first monitored MyD88^{FL} (WT) with HS-AFM on an APTES (3-aminopropyltriethoxysilane)-modified mica stage. Most of the MyD88^{FL} molecules were visualized as single particles (Fig. 6a). However, their surfaces are not smooth but have two humps, as clearly shown in Z-slice profiles, suggesting the existence of two subparticles tightly bound together to form a “closed” state (Fig. 6c, 7a-c). We assumed the two humps to correspond to DD_{MyD88} and TIR_{MyD88}, as the size of the particles was approximately consistent with the crystal structures. Notably, domain identification was impossible because of the similar sizes of the particles, with a size difference smaller than the spatial resolution of the measurement. In addition to the two-humped “closed” particles, we also found a subset of tandemly arranged pairs of particles, which changed their relative position from time to time. These particles may correspond to an elongated “open” state of MyD88^{FL}, in which no interdomain interaction occurs (Fig. 6b, d). Notably, the population of the open particles was smaller than that of the closed state.

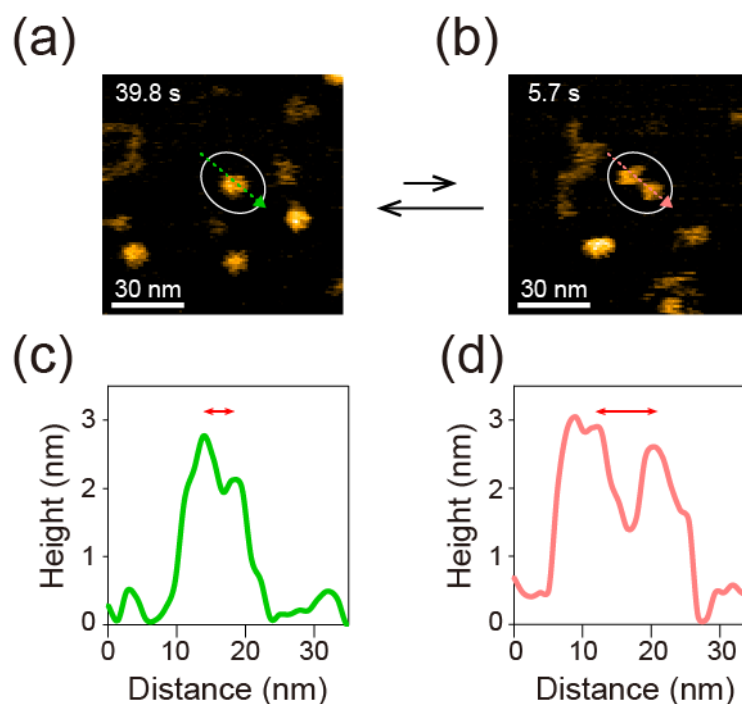


Figure 6 Single-molecule analysis of MyD88^{FL} (WT) with HS-AFM.

(a,b) Representative HS-AFM images of MyD88^{FL} in the closed state (a) and the open state (b) recorded on APTES-modified micas. These HS-AFM images were clipped from measured movie. (c, d) Sectional views of the green and pink arrows in the AFM images in (a) and (b), respectively.

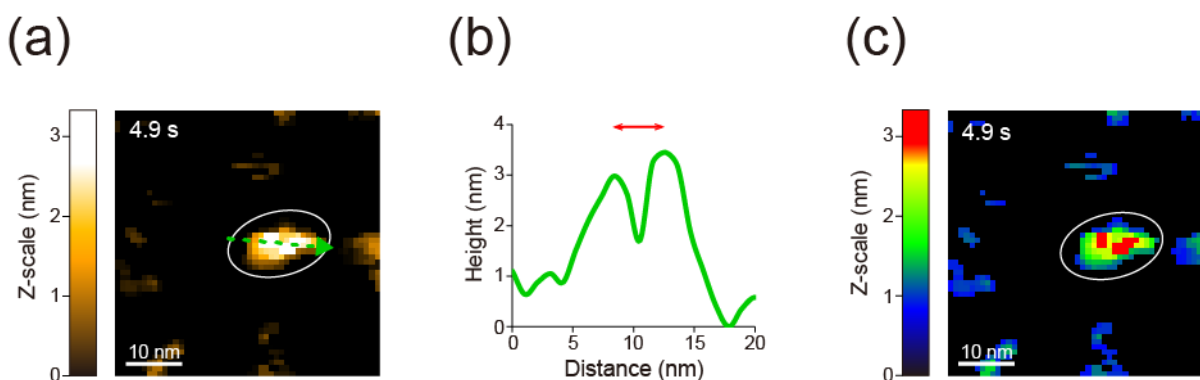


Figure 7 The distinction between two subparticles in the closed state in the AFM image.

(a) Representative HS-AFM images of MyD88^{FL} in the closed state recorded on APTES-modified micas. (Frame rate, 50 ms/frame; scan area, 50 × 50 nm² with 50 × 50 pixels; Z-scale, 4.3 nm; scale bars, 10 nm.) The sectional view of the green arrow in the AFM image (b) and the image with the color scale changed from black-yellow to multicolor (c) show that the image visualized as the single subparticle in (a) consisted of two particles.

3.4 Histogram analysis to identify intermolecular interaction interfaces in MyD88^{FL}

D_{cc} (center-to-center distance between two subparticles or humps, the putative TIR and DD of MyD88^{FL}) was measured for each molecule in the AFM images and found to be distributed between 2 and 9 nm (Fig. 8a, gray)²⁷. The histogram of D_{cc} showed a skew shape and was explained by the summation of two Gaussian distributions with peaks at 3.66 ± 0.02 nm and 5.30 ± 0.04 nm (Fig. 8a, green and pink), which we think correspond to the D_{cc} distributions of the closed and open states, respectively.

Next, to determine whether the DD-TIR interaction in the closed state of MyD88^{FL} was indeed mediated by the key residues identified in our NMR experiments, we examined amino acid substitutions in MyD88^{FL}. We prepared a derivative harboring a substitution in the TIR domain, MyD88^{FL} (RA), in which R217 of TIR_{MyD88} of MyD88^{FL} was replaced with alanine. The AFM images recorded in this way showed a dramatic change in the distance distribution. Specifically, the D_{cc} distribution of MyD88^{FL} (RA) was dominated by a single Gaussian with a peak at 4.70 ± 0.03 nm, although a minor subset was found at 6.91 ± 0.04 nm and was even elongated (Fig. 8a vs. 8c). Importantly, the closed state of MyD88^{FL} (RA) was essentially absent (Fig. 8e, Table 1).

We then created MyD88^{FL} (EQ), in which E52, E53 and D55 in DD_{MyD88} were replaced by Gln or Asn (E52Q-E53Q-D55N), and conducted HS-AMF measurement of the sample. For this measurement, we employed an unmodified mica stage (see Fig. 9). As expected, the distance distribution of MyD88^{FL} (EQ) was best explained by a single Gaussian distribution with a peak at 5.49 ± 0.04 nm (Fig. 8b vs. 8d), indicating that the derivative existed mostly in the open state (Fig. 8f).

In this way, our AMF measurements demonstrated that MyD88^{FL} can adopt two distinct states (open and closed). In addition, the two variants, MyD88^{FL} (EQ) and MyD88^{FL} (RA), in which amino acid residues putatively mediating the DD_{MyD88}-TIR_{MyD88} interaction were mutated, were shown to rarely adopt the closed state. These findings suggest that the anionic sites of DD_{MyD88} and the cationic site of TIR_{MyD88} interact in an intramolecular fashion, most likely through the sites proposed in our NMR experiments

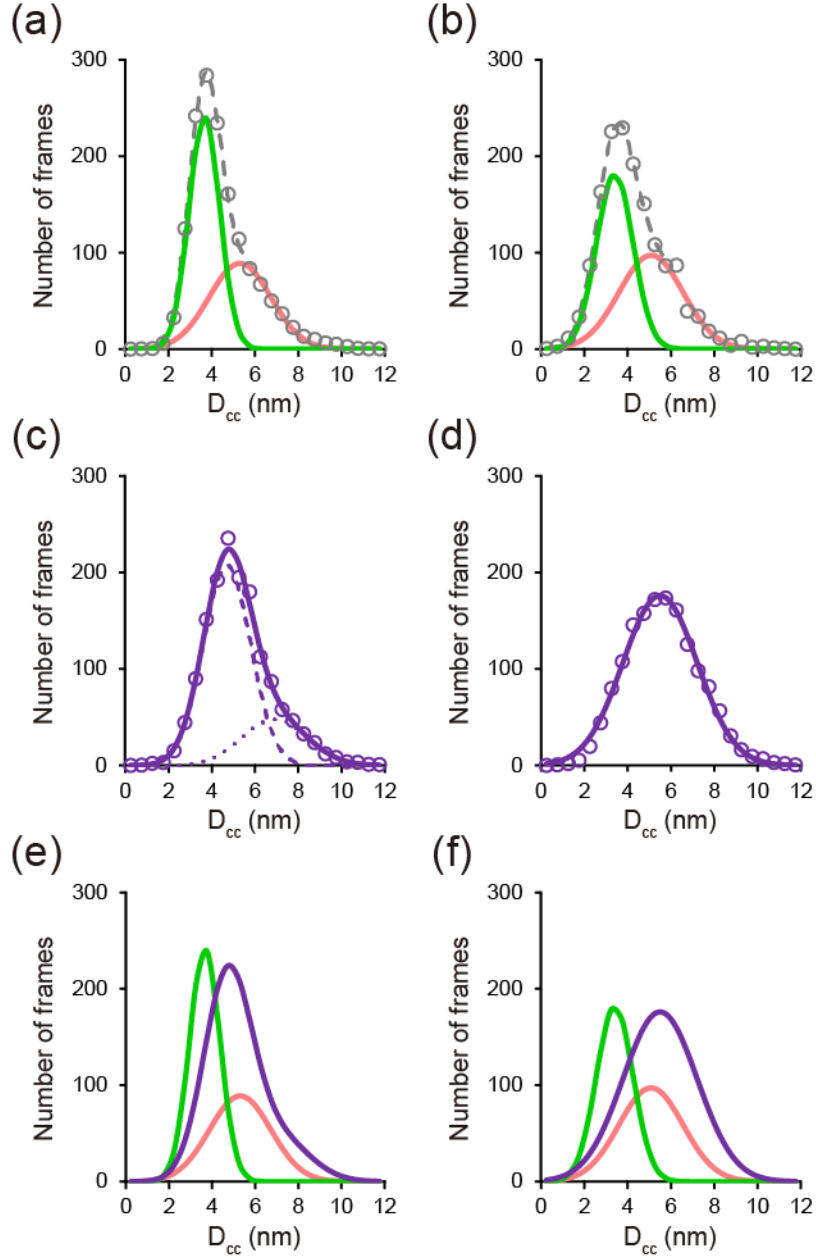


Figure 8 Histogram analysis for MyD88^{FL}.

(a,b) Histogram (open circles) for MyD88^{FL} (WT) recorded on (a) an APTES-modified mica surface and (b) a mica surface. The gray dotted line represents the best fit to the data calculated by the summation of two Gaussian components corresponding to the closed state (green) and the open state (pink). (c) Histogram (open circles) for MyD88^{FL} (RA) recorded on an APTES-modified mica surface. The purple solid line represents the best fit with the summation of two Gaussian components (long broken line and short broken line). (d) Histogram (open circles) for MyD88^{FL} (EQ) recorded on the mica surface. The purple line represents the best single-Gaussian fit. (e,f) Comparison of the fitting curves of histograms. The curves correspond to (e) the APTES-modified mica surface or (f) the mica surface. Both overlaid fitting curves show that the mutants predominantly adopted the open state of MyD88^{FL}.

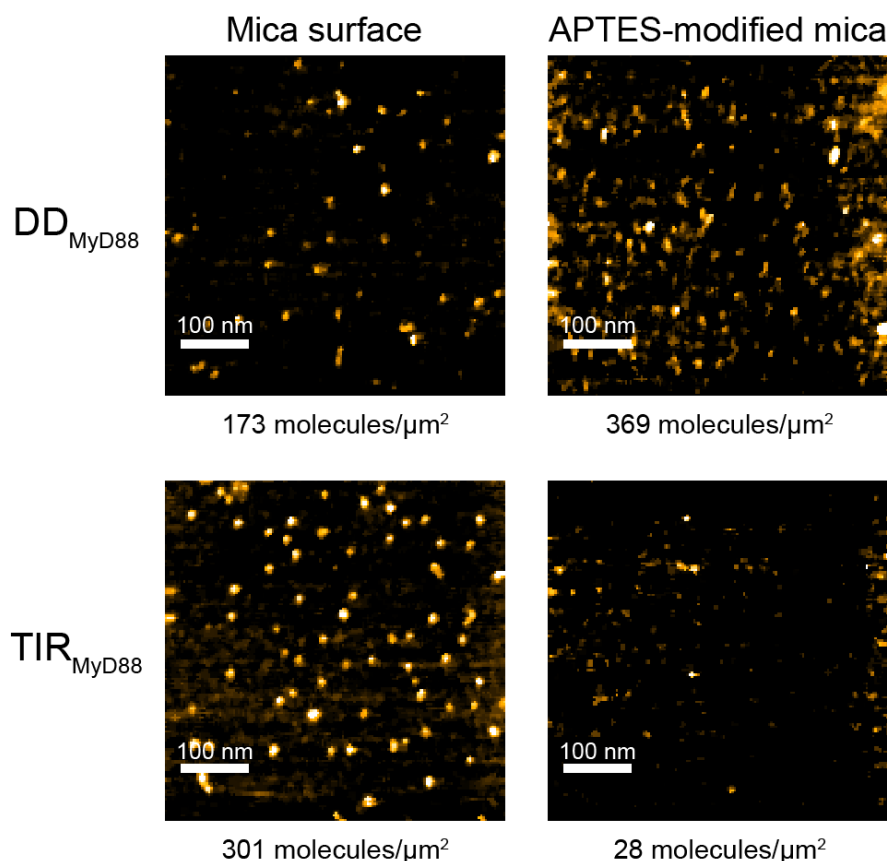


Figure 9 Unmodified and APTES-modified mica surfaces adsorbed DD_{MyD88} and TIR_{MyD88} with different efficiencies.

HS-AFM images recorded with 10 nM isolated DD_{MyD88} on a mica surface (top left) or an APTES-modified mica surface (top right) and with 10 nM isolated TIR_{MyD88} on a mica surface (bottom left) or an APTES-modified mica surface (bottom right). Frame rate, 600 ms/frame; scan area, 500 × 500 nm² with 150 × 150 pixels; Z-scale, 4.5 nm; scale bars, 100 nm.

In AFM experiments, protein samples need to be weakly attached to AFM stages, which largely rely on electrostatic adsorption onto the surface of the stages. Considering the isoelectric points of DD_{MyD88} (theoretical pI = 4.57) and TIR_{MyD88} (theoretical pI = 8.97), either of the two domains in MyD88^{FL} may dominantly attach to the stage depending on the electrostatic properties of the surface. This situation may cause inhomogeneous attachment at the molecular level on the surface, which can generate artefacts, especially in experiments using a series of MyD88 derivatives, since amino acid substitutions may change the charges of the domains, altering the adsorption mode of the molecules.

To alleviate such artefacts, we employed two different AFM stages, unmodified mica and mica modified with APTES, for different MyD88^{FL} derivatives. The former is negatively charged, while the latter is positive. Hence, as shown in the figures above, the APTES-modified mica adsorbed more DD_{MyD88}, while the unmodified mica adsorbed more TIR_{MyD88}. Thus, we used the former for examining the MyD88^{FL} derivatives harboring substitutions in TIR_{MyD88}, as the surface is expected to adsorb DD_{MyD88} of MyD88^{FL}, leaving TIR_{MyD88} of MyD88^{FL} free to move regardless of whether substitutions are introduced. For a similar reason, we used unmodified mica to examine substitutions in DD_{MyD88} of MyD88^{FL}, as the surface is expected to adsorb TIR_{MyD88} of MyD88^{FL}, leaving DD_{MyD88} mobile. It should be noted that the HS-AFM results for MyD88^{FL} (WT) recorded on these two distinct surfaces were indeed consistent with each other (see Fig. 8a and 8b).

Table 1. Summary of the histogram analysis.

State	Stage	D_{cc} (nm)	Number of molecules analyzed	Number of frames analyzed
Closed state of MyD88 ^{FL} (WT)	Mica surface	3.40 ± 0.02	30	1500
Open state of MyD88 ^{FL} (WT)	Mica surface	5.08 ± 0.04		
MyD88 ^{FL} (EQ)	Mica surface	5.49 ± 0.04	30	1500
Closed state of MyD88 ^{FL} (WT)	APTES-modified mica	3.66 ± 0.02	30	1500
Open state of MyD88 ^{FL} (WT)	APTES-modified mica	5.30 ± 0.04		
Dominant state of MyD88 ^{FL} (RA)	APTES-modified mica	4.70 ± 0.03	30	1500
Minor state of MyD88 ^{FL} (RA)	APTES-modified mica	6.91 ± 0.04		

D_{cc} values are shown as the mean $\pm$ SEM.

4. Discussion

4.1 Auto-suppression mechanism

In this study, we have demonstrated the intramolecular DD-TIR interaction of MyD88. In fact, another TIR-containing signaling adaptor protein, TRIF (TIR domain-containing adaptor molecule-inducing interferon- β), has been proposed to adopt an autosuppressed form, in which its N-terminal globular domain is presumed to bind TIR_{TRIF} in an intramolecular fashion that prevents the homodimerization of the TIR_{TRIF}, which is a necessary step for downstream signaling^{28–30}. Such a scenario is also possible in MyD88 because the intramolecular DD_{MyD88}-TIR_{MyD88} interaction can suppress DD-DD oligomerization, as the sites on DD_{MyD88} for binding to TIR_{MyD88} identified in our study are also involved in the DD_{MyD88}-DD_{IRAK4} interaction in the Myddosome. Namely, the adoption of the closed state and the formation of the Myddosome seem to be mutually exclusive for MyD88. In other words, the closed state of MyD88 likely corresponds to its dormant form, whose activation is triggered only when the receptors dimerize and its interaction surface for TIR_{MyD88} forms a suitable shape.

4.2 Pathogenic mutations in TIR_{MyD88}

Tumor genetic mutation analyses of liquid biopsies have identified gain-of-function mutations of MyD88, some of which are frequently found in lymphomas, such as the activated B-cell-like subtype of diffuse large B-cell lymphoma³¹, in which NF- κ B and JAK kinase signaling were revealed to be constitutively activated, contributing to the proliferation and survival of malignant cells. One example of such a mutation is L265P (also annotated as L252P). Studies including molecular simulations^{9,32,33} suggested that the mutation promotes the spontaneous oligomerization of TIR_{MyD88} (L265P), which can consequently cause the oligomerization of DDs and thus Myddosome formation. In this case, receptor activation is not necessary to trigger signaling, which is consistent with the constitutive activation observed in the reported lymphomas^{31,34,35}. In agreement with this notion, we also found a strong tendency toward aggregate formation by isolated TIR_{MyD88} when the L265P mutation was introduced (Fig. 10). Thus, the intramolecular DD_{MyD88}-TIR_{MyD88} interaction observed in our study may be weaker than the TIR_{MyD88}-TIR_{MyD88} interaction once the L265P

mutation is introduced. Hence, the closed state cannot be maintained, and the TIR_{MyD88} oligomers start to form the Myddosome. In addition, we propose an alternative mechanism: the L265P mutation may substantially weaken the DD_{MyD88}-TIR_{MyD88} interaction, making the dormant closed state much less populated and hence stimulating DD_{MyD88} oligomerization as well as TIR_{MyD88} oligomerization for Myddosome formation. This idea is well supported by the strong tendency of DD_{MyD88} to form active oligomers *in vitro* (Fig. 3) and in cells³⁶.

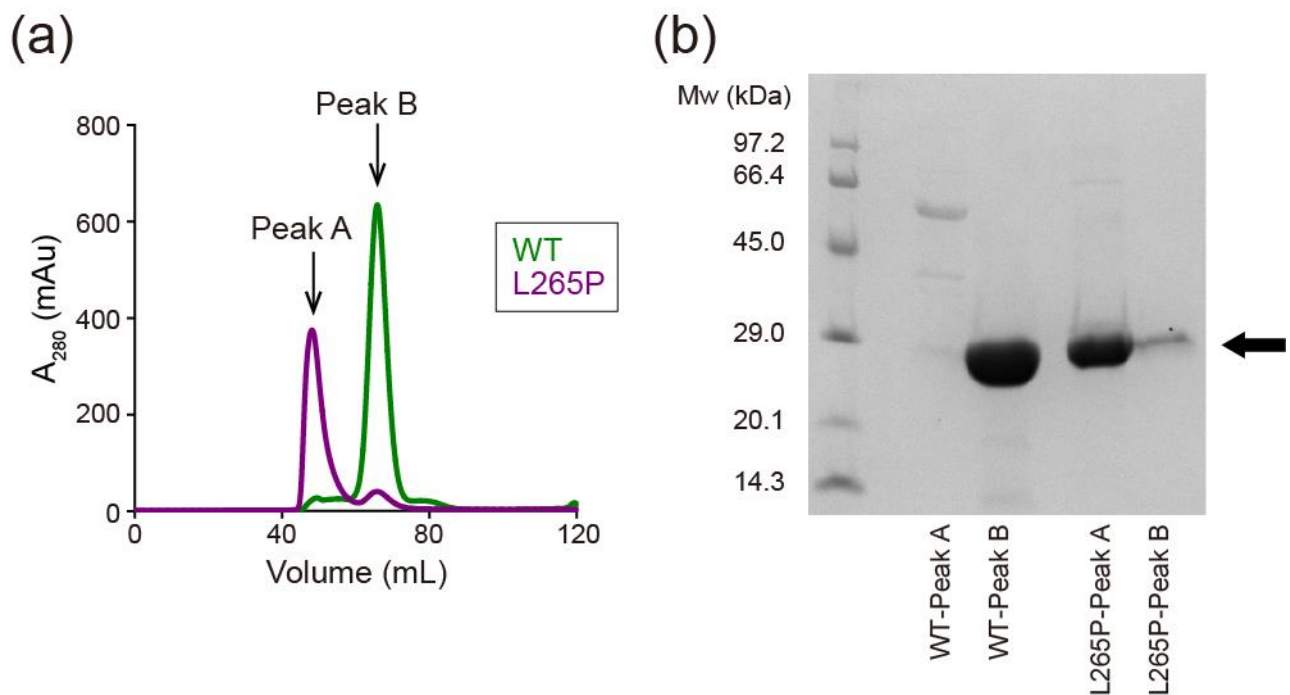


Figure 10 His-GB1-TIR_{MyD88} aggregation shown by chromatography.

(a) Size-exclusion chromatograms of His-GB1-TIR_{MyD88} (WT) (green) and His-GB1-TIR_{MyD88} (L265P) (purple). The His-GB1-TIR_{MyD88} (L265P) eluted essentially in the void region, suggesting that the protein was mostly aggregated. (b) SDS-PAGE analysis showed that most of the His-GB1-TIR_{MyD88} (L265P) eluted in Peak A, while His-GB1-TIR_{MyD88} (WT) eluted in Peak B. The position of Peak B corresponds to monomeric His-GB1-TIR_{MyD88}.

Consistently, although endogenous MyD88^{FL} is inactive as long as its upstream receptor is not activated, ectopic overexpression of MyD88^{FL} was reported to constitutively activate its downstream signaling³⁷. This result suggested that, at a physiological concentration of MyD88^{FL}, the intramolecular DD_{MyD88}-TIR_{MyD88} interaction is dominant³⁸, resulting in a higher proportion of the closed state. However, at higher concentrations, the intermolecular interaction becomes dominant³⁹. Moreover, our TCS-NMR

experiments showed that R264 (R251) in TIR_{MyD88} resided at the binding interface for DD_{MyD88}. This residue is immediately next to L265 (L252), and hence, the L265P mutation can cause substantial changes in the shape of the DD_{MyD88}-TIR_{MyD88} interface. Intriguingly, another lymphoma-related mutation, M232T (M219T)³¹, also occurs near this region (Fig. 11). These findings support the idea that the constant exposure of free DD_{MyD88} in the cytosol as a result of such mutations contributes at least partially to the constitutive activation of MyD88-dependent signaling.

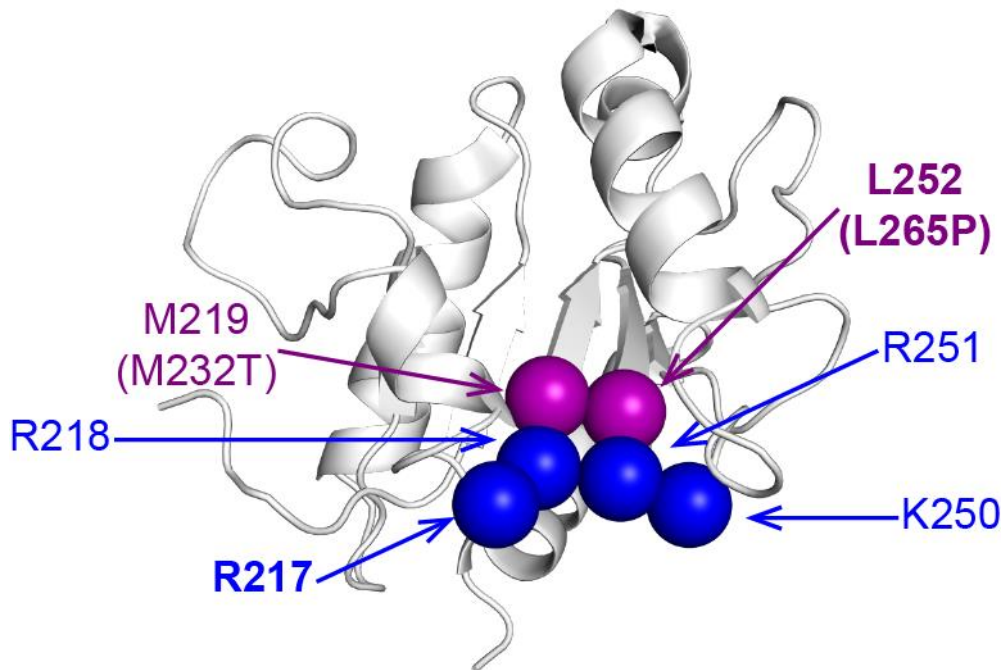


Figure 11 Position of pathogenic mutations and the intramolecular interaction site on TIR_{MyD88}.

Two mutations of TIR_{MyD88}, M232T and L265P (purple spheres), were reported as pathogenic mutations in lymphomas³¹. These mutated residues neighbor four cationic residues (R217, R218, K250 and K251) identified in this study as the intramolecular DD-TIR interaction site of MyD88 (blue spheres). Their proximity implies that the pathogenic mutations could abolish the intramolecular DD-TIR interaction of MyD88, contributing to the constitutive activation of MyD88-dependent signaling as observed in the reported lymphomas.

5. Conclusion

In this study, we demonstrated the DD-TIR intramolecular interaction of MyD88 by using NMR spectroscopy and HS-AFM. Real-time monitoring by AFM revealed that MyD88^{FL} can adopt the closed form, in which DD_{MyD88} and TIR_{MyD88} are tightly bound together, which allowed us to propose the autosuppression mechanism for the molecule (Fig. 12). Tight regulation implemented by such a mechanism would be critical because the unregulated activation of MyD88-dependent signaling can lead to innate immune and inflammatory disorders that severely damage organisms.

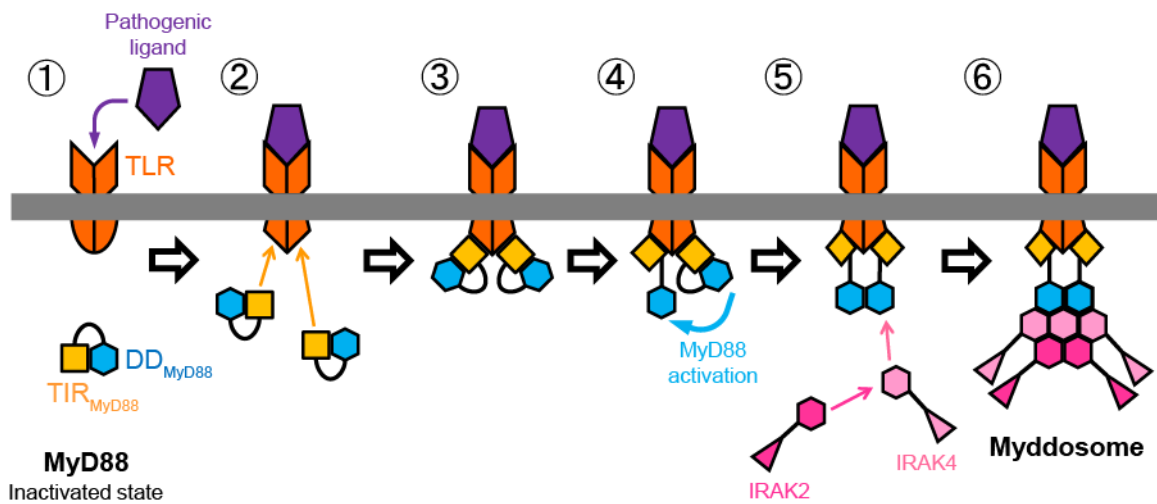


Figure 12 The autosuppression model of MyD88.

1) Without ligand stimulation, MyD88 mostly exists in the closed state, in which both TIR_{MyD88} dimerization and DD_{MyD88} oligomerization with DD_{IRAKs} are strongly suppressed. 2-3) Once TIR_{TLR} forms an active dimer, MyD88 is recruited to and accumulated under the plasma membrane via the TIR-TIR interaction²². As the local concentration of MyD88 increases, the molecules become open and initiate the formation of DD-DD oligomers via intermolecular interactions. 5-6) DD_{IRAKs} is recruited to yield the Myddosome complex. When the L265P mutation (or other analogous mutations) occurs in MyD88, TIR_{MyD88} gains a strong aggregation propensity, leading to the spontaneous formation of a dimer/oligomer. In addition, the closed state may be destabilized by the mutation. Both of these mutation outcomes promote Myddosome formation even without receptor dimerization, which would constitutively activate downstream signaling, as reported, contributing to tumor survival.

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Chapter III

Structural analysis of homo-oligomers
of the TIR domain of MyD88

Chapter III

Structural analysis of homo-oligomers of the TIR domain of MyD88

Section I

High-speed AFM analysis of the annular complex of the MyD88 TIR domain

Abstract

MyD88 (myeloid differentiation factor 88) is an essential adaptor protein in the signaling of the Toll-like and interleukin-1 receptor superfamily. MyD88 comprises two functional domains, DD_{MyD88} (death domain) and TIR_{MyD88} (Toll/interleukin-1 receptor domain). Each of these domains forms homo- or hetero-oligomers, which are critical for mediating downstream signaling. Here, to gain insights into the structure and dynamics of oligomeric states of TIR_{MyD88}, we employed HS-AFM (high-speed atomic force microscopy) for direct visualization. At high concentrations ($>10\ \mu\text{M}$), TIR_{MyD88} assembled into two-stranded fibrils on the AFM stage. To our surprise, the fibrils adopted an annular shape. Despite its seeming dissimilarity, the molecular arrangement inside was likely compatible with a previous study. We also monitored the dynamics of the fibrils and the disintegration and regeneration over time, stimulating further exploration of the dynamics of oligomerization of TIR_{MyD88} as well as its functional relatives in the innate immune signals with HS-AFM.

1. Introduction

MyD88 (myeloid differentiation factor 88) is an intracellular adaptor protein that functions in the innate immunity and inflammation¹, by bridging activated membrane receptors of the TLR (Toll-like receptor)/IL-1R (interleukin-1 receptor) superfamily and cytosolic kinases called IRAKs (interleukin-1 receptor-associated kinases). MyD88 comprises two protein-protein interaction modules: DD_{MyD88} (death domain) at the N-terminus and TIR_{MyD88} (Toll/IL-1R domain) at the C-terminus. These domains are connected by an ~35 amino acid residue linker^{2,3}, where we recently proposed an autosuppression mechanism via the intramolecular DD_{MyD88}-TIR_{MyD88} interaction in the resting state⁴.

For instance, during the TLR4 signaling, a lipopolysaccharide comprised in pathogenic bacteria binds the extracellular domain of TLR4 with cofactors, inducing homodimerization of the both extracellular and cytosolic regions of TLR4 to dimerize TIR_{TLR4} (TIR domain of TLR4) underneath the membrane⁵. The dimerized TIR_{TLR4} utilizes Mal/TIRAP (MyD88 adapter-like/TIR-containing cytosolic adaptor proteins) to transduce the signaling via MyD88⁶. This process allowed MyD88 to accumulate under the plasma membrane and increase its two-dimensional concentration to accelerate activation of the autoinhibited molecules via intermolecular interactions⁴. After unlocking by the signaling receptor, MyD88 starts to recruit IRAK4 and IRAK1/2 further to assemble a large protein complex termed the Myddosome, which is mediated by oligomeric interactions between the DDs of MyD88, IRAK4 and IRAK1/2³. The Myddosome formation results in the activation of KDs (kinase domains) of IRAK1/2, which eventually activates transcription factors NF- κ B/AP-1 to produce inflammatory cytokines such as IL-1 β , IL-6, and TNF- α ⁷.

The crystal structure of DD_{MyD88}-DD_{IRAK4}-DD_{IRAK2} hetero-oligomer described its helical oligomeric architecture³, providing a clear picture of the oligomeric assembly functioned as a scaffold to promote auto-phosphorylation of IRAKs. However, although monomeric structures of TIR_{MyD88} and other TIR-containing adaptor proteins have been reported⁸⁻¹¹, the details of the molecular mechanism to form dimers/oligomers have not been well understood. TIR_{MyD88} is a globular protein with a diameter of approximately 3 nm, and TIR_{MyD88} has multiple binding interfaces that interact with various proteins (Fig. 1a)^{4,8}. Numerous biochemical studies

of TIR_{MyD88} were conducted to identify sites on its surface that are critical for the dimeric/oligomeric association, based on proposed structural models¹².

Recently, TIR_{Mal} (TIR of Mal/TIRAP) was shown to form a fibril *in vitro*, in which 12 protofilaments assembled into a hollow tube¹³, and the molecular structure and intermolecular interfaces in the fibrils were revealed by cryo-EM analysis, allowing us to envisage the functional TIR-TIR interactions in cells. In the report, TIR_{MyD88} was also shown to form fibrils when seeded with a small amount of TIR_{Mal}, although it did not on its own. Because elongation of the TIR_{MyD88} fibrils required only a fractional amount of seeding TIR_{Mal}, the resultant fibrils were primarily made up of TIR_{MyD88}. Unlike the TIR_{Mal} fibrils, the TIR_{MyD88} fibrils displayed substantial heterogeneity in width and shape. The authors proposed that these fibrils were also formed from two-stranded head-to-tail arrays of TIR_{MyD88} in a similar manner to those of TIR_{Mal}. In this study, to gain further insights into the structure and dynamics of homo-oligomers formed by TIR_{MyD88}, we directly visualized the domain by using HS-AFM (high-speed atomic force microscopy).

2. Material and Method

2.1. Construction of expression vectors

The TIR_{MyD88} expression vector was prepared and described in the previous study⁴. The construct was cloned in a pET-28a vector [Novagen, WI, USA] as the N-terminal 6×His-tagged GB1 (streptococcal protein GB1 domain, residues 261-324) fusion, followed by a HRV3C (human rhinovirus 3C protease) site and the coding region of TIR_{MyD88} (MyD88, NM_002468.4, residues 166-309, annotated as 153-296 in this study).

2.2. Protein expression and purification

E. coli BL21(DE3) cells were transformed with the expression vector for the His-GB1-tagged TIR_{MyD88} and grown in LB medium. After induction of protein expression, TIR_{MyD88} was extracted and purified as previously described⁴. Briefly, His-GB1-TIR_{MyD88} was first purified with His-tag affinity column chromatography [Roche, Basel, Switzerland]. After removal of the His-GB1 tag with HRV3C, the protein was further purified with cation exchange chromatography and size-exclusion chromatography [GE, IL, USA]⁴.

2.3. HS-AFM imaging

A HS-AFM was equipped with a small cantilever (BL-AC10-DS-A2 [Olympus, Tokyo, Japan]: spring constant, $k = 0.1$ N/m, resonance frequency, $f = 400\sim 500$ kHz in water) and was operated in tapping mode at room temperature^{14–16}. An amorphous carbon tip was grown on the top of each cantilever by electron-beam deposition with a scanning electron microscope (ERA-8000FE [Elionix, Tokyo, Japan]). The free oscillation amplitude was 1.4~2 nm, and the typical set-point amplitude was 85% of the free oscillation amplitude. A mica disk (1 mm in diameter) fixed by epoxy glue on a glass rod (2 mm in diameter and 2 mm in height) was used as a sample stage¹⁵. Before or after the sample stage was fixed by nail polish on the z-piezo of the HS-AFM scanner, the mica was freshly cleaved. Freshly cleaved mica was used as a substrate. Then, 5 μ L of 10 or 100 μ M TIR_{MyD88} was adsorbed on the substrates in AFM sample buffer containing 20 mM HEPES-NaOH (pH 7.4), 0.5 mM EDTA and 10 mM DTT. After 10 min, the sample was rinsed with 20 μ L of sample

buffer. The sample stage was filled with 63 μL of sample buffer before starting measurement, and 7 μL of sample buffer, 100 μM TIR_{MyD88} or 0.1 mg/mL anti-MyD88 C-terminal antibody, PRS2127 [Sigma-aldrich, MO, USA] was added to the sample stage after starting measurements.

2.4. Processing of HS-AFM data

The HS-AFM image sequences were processed using two laboratory-developed software, Kodec 4.5.7.25 coded in Visual C# of Visual Studio 2010 [Microsoft, WA, USA]¹⁷ and a software based on Igor Pro 6.36 [Wave Metrics Inc., OR, USA] [17]. The background height level was adjusted to zero after background leveling in all images¹⁸. After taking images, we tracked a target molecule using two-dimensional (2D) correlation analysis to compensate for the slow drift of the sample stage position in the x- and y-directions. A 3×3 pixel-average filter was applied to each tracked image to reduce noise¹⁹. To generate a frame-averaged image, successive frames (~ 20 frames) were stacked and averaged. The collected data were processed with Excel 2010 [Microsoft, WA, USA].

3. Result

3.1. The observation of TIR_{MyD88} homo-oligomer

Purified TIR_{MyD88} molecules were incubated on a mica substrate for 10 min. The substrate was then immersed in a buffer solution and imaged with a HS-AFM^{15,20}. The obtained images showed that the TIR_{MyD88} particles had formed fibrils on their own on the mica substrate. To our surprise, the shape of the fibrils was very characteristic; many of them were annularly shaped, forming large ring structures (Fig. 1b, 1c) with the average height and diameter of 6.6 nm and 26 nm, respectively. However, diameters deviated from approximately 24 nm to 28 nm, whereas heights converged near 6.6 nm. Most rings were almost uniform, though there were rings with some deviations in diameter, suggesting that rings were constituted from specific homotypic interactions of TIR_{MyD88} rather than via nonspecific association of the molecules. Considering the molecular diameter of TIR_{MyD88} , which is ~ 3 nm (Fig.1a), the ring height corresponds to two molecules of TIR_{MyD88} , suggesting that the ring presumably consisted two layers of TIR_{MyD88} molecules. It should be noted that fibrils did not form at all, even when incubating the sample at a lower concentration ($<1 \mu\text{M}$). This observation was consistent with our recent AFM study, in which full-length MyD88 and TIR_{MyD88} appeared as monomers at 10 nM ⁴.

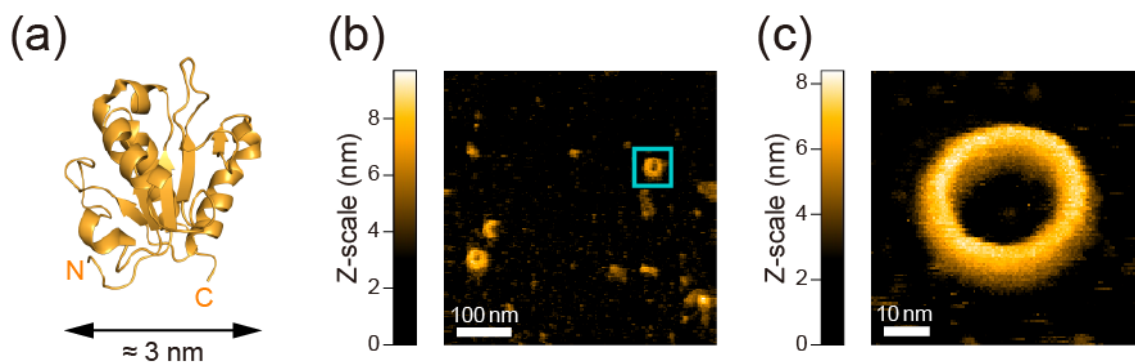


Figure 1 AFM images of the Ring.

(a) The NMR structure of monomeric TIR_{MyD88} (PDB ID: 2Z5V). (b) The representative overview image of TIR_{MyD88} rings on a mica surface. (Frame rate, 600 ms per frame; scan area, $500 \times 500 \text{ nm}^2$ with 150×150 pixels; Z-scale, 9.7 nm; scale bars, 100 nm.) (c) The zoomed image in the cyan square of (b). (Frame rate, 600 ms per frame; scan area, $60 \times 60 \text{ nm}^2$ with 120×120 pixels; Z-scale, 8.4 nm; scale bars, 10 nm.)

The measured movie shows the dynamics of the rings; some disintegrated over time, while others regenerated. Sectional views taken from partially formed rings showed three distinct regions with different heights of 6.6 nm, 3.2 nm, and less than 2.0 nm (Fig. 2). The 6.6 nm height corresponds to double the diameter of TIR_{MyD88}, indicating that two layers existed in the region. The 3.2 nm corresponds to the diameter of TIR_{MyD88}, meaning that in the region, the upper layer of the ring was absent, and only the lower layer existed. In the region with < 2.0 nm height, both layers were absent. Considering these height distributions and shapes of the partially formed ring, it is likely that the ring structure adopts a rotational symmetric bilayer and does not have helical symmetry.

For further analysis, we picked up one ring, to which rigorous image processing (frame average using successive 21 frames) was applied¹⁹. The resultant image allowed us to identify nodules on the surface of the ring structure (Fig. 3a), and each of these nodules presumably corresponded to one copy of TIR_{MyD88}. Because the number of nodules was 29 (Fig. 3b), we presumed that this ring consisted of 58 TIR_{MyD88} molecules, adopting a 29-fold rotational symmetric bilayer structure (Fig. 3c); however, there would be some deviation in different rings because there was some deviation in their diameter.

The TIR_{Mal} fibril was reported to adopt a hollow tube structure with inner and outer layers with diameters of 13 and 25 nm, respectively¹³. The diameter of our rings (~26 nm) was similar to but slightly larger than the outer layer of the TIR_{Mal} fibrils. However, the thickness of rings corresponded to only a single layer of molecules. In addition, our ring structure did not have helical symmetry but was a rotational symmetric oligomer, while the TIR_{Mal} fibrils had helicity. Thus, the molecular arrangements in these tube-like structures seem to have some differences with each other, despite their superficial similarity in shape (tube and ring). In the previous study, fibril formation of TIR_{MyD88} was observed only when seeded with TIR_{Mal}, but the fibrils in EM images displayed more heterogeneity than that of TIR_{Mal} with mostly much larger widths than the diameter of our rings¹³. In contrast, we did not see such fibrils in our AFM measurement.

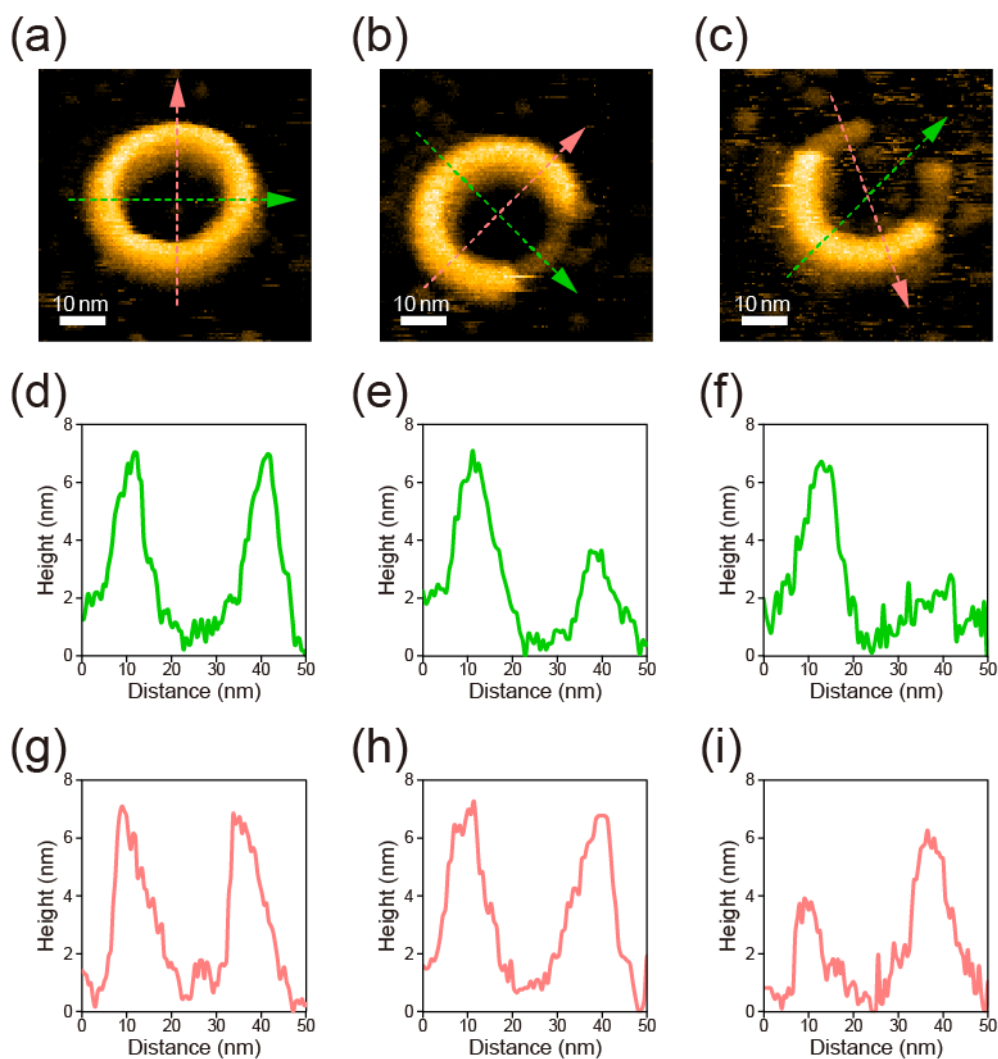


Figure 2 Single-molecule analysis of the Ring.

(a-c) Representative HS-AFM images of a ring in the dissociation process. Each image was recorded at 13.84 s (a), 17.24 s (b) and 49.40 s (c) in the measured movie. (Frame rate, 600 ms per frame; scan area, $60 \times 60 \text{ nm}^2$ with 120×120 pixels; Z-scale, 8.4 nm; scale bars, 10 nm.) (d-i) Sectional views of the green and pink arrows in the AFM images in (a), (b) and (c), respectively. Sectional views showed that the diameter and height of the ring were approximately 28 nm and 6.5 nm in a perfect state (d), (g), respectively. In the partially lacking state, the heights of lacking points were approximately 3.2 nm (e) or less than 2 nm (f).

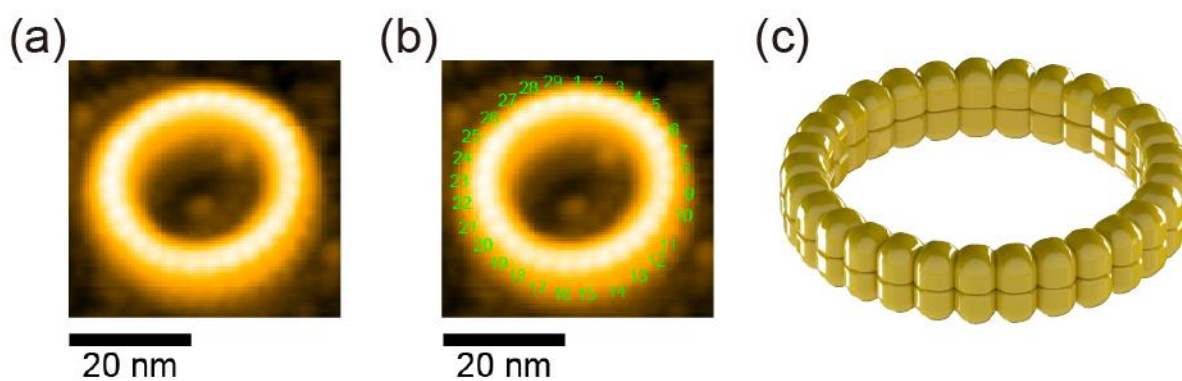


Figure 3 Nodule identification in the processed image.

(a) The image of Ring of Fig. 1c processed by frame averaging. (b) Numbering of nodules comprised in Ring. (c) Schematic of the Ring from overlook view.

3.2. Investigations into detail of Ring

To investigate the molecular orientation of TIR_{MyD88} in the ring, we then performed an experiment in which an anti-MyD88 antibody, which binds the MyD88 C-terminal (279-296) peptide epitope, was added to rings preformed on the AFM stage (Fig. 4a, 4b). Some antibody molecules were located on rings (Fig. 4c), suggesting that the C-terminal epitope region of TIR_{MyD88} was plausibly exposed in the ring structure.

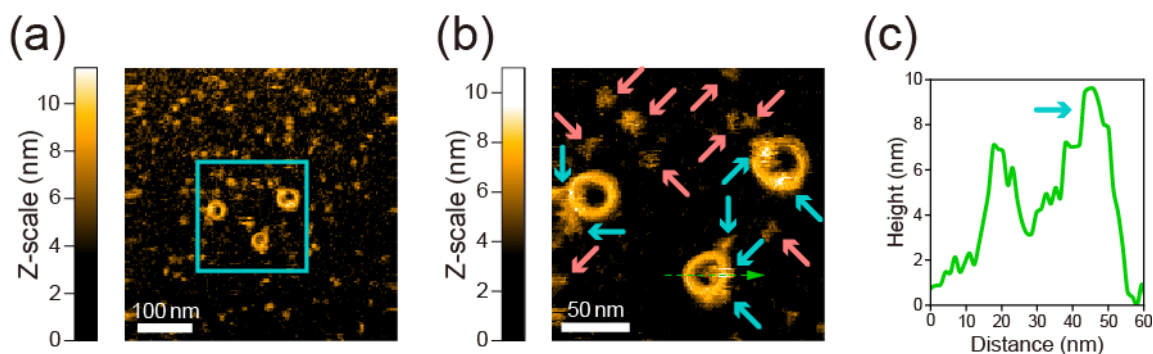


Figure 4 AFM images of the Ring in the presence of antibody molecule.

(a) The representative overview image of rings on a mica surface in the presence of 10 $\mu\text{g/mL}$ anti-MyD88 C-terminal antibody. (Frame rate, 600 ms per frame; scan area, $500 \times 500 \text{ nm}^2$ with 150×150 pixels; Z-scale, 11.5 nm; scale bars, 100 nm.) (b) The zoomed image in the cyan square of (a). Cyan and pink arrows indicate antibody on a ring and mica surface, respectively. (Frame rate, 600 ms per frame; scan area, $200 \times 200 \text{ nm}^2$ with 150×150 pixels; Z-scale, 11.0 nm; scale bars, 50 nm.) (c) The sectional view of the green broken arrow in the AFM images in (b). The right peak indicated by the cyan arrow is an antibody molecule located on the ring.

4. Discussion

4.1. Discussion about artifacts

For our AFM measurements, samples to be measured need to weakly associate onto a mica surface. This may bring some artificial factors into the measurements, such as the two-dimensional concentration effect, the electrostatic field produced by the mica substrate, and the biased surface exposure of adsorbed molecules^{20–22}. These artificial factors might drive the ring assembly of TIR_{MyD88}. In fact, it was previously shown that fibril formation of TIR_{MyD88} required TIR_{Mal}, indicating that the propensity of TIR_{MyD88} to form fibrils in bulk solution would not be strong. Because the surface of the mica substrate is negatively charged, while TIR_{MyD88} is positively charged (theoretical pI = 8.97) in our measurement conditions, the adsorption of TIR_{MyD88} on the surface may have been promoted via electrostatic attraction. Then, the spatial diffusion of the molecules would be substantially restricted only near the surface, increasing the effective concentration of the molecules at the surface. Such a two-dimensional concentration effect may have played a role in enhancing the homotypic interactions of TIR_{MyD88}. Even if the affinity may have been exaggerated by the concentration effect, the mode of the homotypic interactions in rings would plausibly have functional significance and reflect those in cells. It may be identical or closely related to those observed in the previous work¹³.

It should also be noted that the lipid bilayer cellular membrane, where MyD88 oligomerizes to function, can be thought of as a negatively charged surface similar to the mica surface. Thus, there should be some two-dimensional concentration effect of TIR_{MyD88} in cells at least near the membrane. Thus, homotypic interactions of TIR_{MyD88}, even when in its native environment, would be stronger than those in the *in vitro* bulk solution.

4.2. Comparison with the previous fiber model

Although the TIR domain forms a dimer, its supramolecular complexes were thought to be unstable *in vitro* and only transiently exist in cells, unlike death-domain superfamily proteins^{13,23}. However, previous research suggested that TIR_{TLR4}, TIR_{Mal}, and TIR_{MyD88} could assemble into supramolecular complexes at high concentrations¹³. According to the report, TIR_{Mal} assembled into linear proto-filaments, 24 copies of which

were circularly arranged based on two distinct homotypic interactions to yield the filamentous complexes. Intriguingly, neither TIR_{TLR4} nor $\text{TIR}_{\text{MyD88}}$ could independently form fibrils. Instead, both TIR_{TLR4} and $\text{TIR}_{\text{MyD88}}$ needed TIR_{Mal} seeding to form fibrils. Although atomic details of the $\text{TIR}_{\text{MyD88}}$ fibrils were not revealed, a model depicting the arrangement of the $\text{TIR}_{\text{MyD88}}$ molecules in fibrils was proposed based on low resolution cryo-EM images by utilizing the structure of the TIR_{Mal} fibrils as a template. Their data suggested two linear strands of $\text{TIR}_{\text{MyD88}}$, each of which is formed via head-to-tail interactions of protomers, laterally assembled together to generate a duplexed proto-fibril. This arrangement is consistent with our two-layered ring structure because it is interpretable as an encircled duplex of two head-to-tail strands of $\text{TIR}_{\text{MyD88}}$. Namely, in the fibril model, the strands were straight; however, in our model, they formed rings. Based on the model structure, the C-terminus of $\text{TIR}_{\text{MyD88}}$ would be exposed, which agrees with our experimental result as the antibody for the C-terminus of $\text{TIR}_{\text{MyD88}}$ was able to bind rings. Nevertheless, as both analyses have limitations in the spatial resolution, we cannot rule out the possibility that molecular arrangements inside those two fibrils are considerably different from each other.

4.3. Consideration the role of Ring in Myddosome formation

It is believed that $\text{TIR}_{\text{MyD88}}$ assembles into homo-oligomers in cells, which leads to Myddosome formation that evokes downstream signaling. For example, Fekonja and colleagues demonstrated that expression of artificially generated stable $\text{TIR}_{\text{MyD88}}$ dimers caused its self-aggregation in the cytosol²⁴, and Latty and colleagues reported that Myddosomes formed via activated TLR4 containing six or more MyD88 molecules⁵. Despite its critical importance in regulating signals from most TLRs and IL-1R family members, analysis of the oligomeric structure of $\text{TIR}_{\text{MyD88}}$ has been hampered for years because of its weak affinity for homotypic interactions. In fact, the isolated $\text{TIR}_{\text{MyD88}}$ prefers to exist as monomers in solution rather than oligomers⁸. In the current study, we found that the ring of $\text{TIR}_{\text{MyD88}}$ formed spontaneously. Such ring structures may not be stably maintained as a whole in cells because of the relatively weak affinity of the homotypic interactions as well as the much more inhomogeneous and complex environment in the cytoplasm. However, the homotypic interaction forming rings should be effective in cells, as our measurements were conducted in

near physiological buffer at room temperature and normal pressure. Furthermore, the mica substrate might mimic the membrane surface to some extent. Thus, it is highly likely that a part of the ring, if not a whole ring, would be formed in cells. Inspection of cells expressing fluorescently labeled MyD88 with super-resolution fluorescence microscopy may resolve this issue.

5. Conclusion

In this study, we demonstrated by using HS-AFM that TIR_{MyD88} can form an annular complex, which has a 29-fold rotational symmetry with a bilayer structure. Because the measurement was conducted under near physiological conditions, the partial structure of the complex should reflect homo-oligomers of TIR_{MyD88} in cells that serve as scaffolds for DD_{MyD88} oligomerization, a critical step in Myddosome assembly. Although the spatial resolution of HS-AFM was not sufficient to fully describe atomic details of the oligomers, ongoing ring disintegration and regeneration were clearly visualized, stimulating further HS-AFM exploration of the dynamic oligomerization process of TIR_{MyD88} as well as its relatives in combination with high resolution structural visualization techniques, such as X-ray crystallography and cryo-EM.

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Chapter III

Structural analysis of homo-oligomers of the TIR domain of MyD88

Section II

Cryo-EM analysis of the tube-like filament of the MyD88 TIR domain

Abstract

In the previous section, we demonstrated by using HS-AFM that TIR_{MyD88} can form the annular complex. Here, we employed cryo-EM to further analyze oligomers of TIR_{MyD88} for structural details. Surprisingly, the cryo-EM images of TIR_{MyD88} demonstrated that TIR_{MyD88} assembled into filament-like polymers rather than the annular complex, and the fibrous oligomer comprised the annular complex as repeating units. Although the analysis could not reach to reveal molecular structure at atomic resolution, this larger homo-oligomer assembly of TIR_{MyD88} allowed us to speculate molecular mechanism of TIR_{MyD88}.

1. Introduction

In the previous section, HS-AFM (high-speed atomic force microscopy) imaging showed that TIR_{MyD88} assembled into the annular complex, which has 29-fold rotational symmetry and bilayer structure. But, further details of the ring structure are still unclear. Moreover, it is impossible to reveal molecular structure of the ring at atomic resolution with HS-AFM, because AFM can only visualize molecular surface. Although a report presented the crystal structure of TIR_{MyD88}¹, the crystal packing was not compatible with the ring structure. To obtain more structural details about the oligomer of TIR_{MyD88}, we employed DLS (Dynamic Light Scattering) and negative stain TEM (transmission electron microscopy). Moreover, we aimed to reveal molecular structure of the ring by single particle analysis with cryo-EM.

2. Material and Method

2.1. Construction of expression vectors

The TIR_{MyD88} expression vector was prepared and described in the previous section. The constructs for the derivatives, such as the alanine-substituted derivatives, were prepared by site-directed mutagenesis.

2.2. Protein expression and purification

For experimental samples, the expression vector was transformed into *E. coli* BL21(DE3) cells. The cells transformed with His-GB1-tag fusion TIR_{MyD88} vector were grown in LB medium. The His-GB1-tag fusion TIR_{MyD88} was first purified with His-tag affinity column chromatography [Roche, Basel, Switzerland]. After removal of His-GB1-tag with HRV3C (human rhinovirus 3C protease), the protein was further purified with cation exchange chromatography and size-exclusion chromatography [GE, IL, USA] with SEC-buffer containing 20 mM potassium phosphate (pH 6.0), 150 mM sodium chloride, and 10 mM dithiothreitol.

His-GB1-TIR_{MyD88} (WT) was purified via similar protocol without removal of His-GB1 tag with GST-HRV3C and cation exchange chromatography. And His-GB1-TIR_{MyD88} (WT) was purified with size-exclusion chromatography with Arg-Buffer containing 20 mM potassium phosphate (pH 7.0), 150 mM sodium chloride, 400 mM arginine chloride, and 10 mM dithiothreitol. His-GB1-TIR_{MyD88} (L252P) was expressed as inclusion bodies. The washed cell debris was resuspended with denaturation buffer containing 50 mM Tris-HCl (pH 8.0), 300 mM sodium chloride and 6 M guanidine chloride. The denaturated His-GB1-TIR_{MyD88} (L252P) was purified with affinity column chromatography. Eluted His-GB1-TIR_{MyD88} (L252P) was refolded by the rapid dilution method with ten times volume dilution buffer containing 20 mM potassium phosphate (pH 7.0), 150 mM sodium chloride, 400 mM arginine chloride, and 20 mM dithiothreitol. Refolded His-GB1-TIR_{MyD88} (L252P) was further purified with size-exclusion chromatography with Arg-Buffer.

After size-exclusion chromatography, all prepared samples were concentrated and filtered through 0.22 µm PVDF filter [Merck Millipore, MO, USA] just before the frozen store.

2.3. DLS

DLS data were collected with the DynaPro NanoStar detector [Wyatt Technologies., CA, USA] at 298K. The prepared samples were incubated during 1 day at 277 K and filtered through 0.02 μm Anotop filter [Sigma-Aldrich, MA, USA] just before the first measurement. After the first measurement, measured samples were incubated during 1 day at 277 K. The additional incubated sample was measured as the second measurement without filtration. Data were acquired with an acquisition time of 30 s and a total of 50 (first measurement) or 100 (second measurement) scans. The curve fittings were performed using regularization functionality in DYNAMICS 7.1.7.16 [Wyatt Technologies., CA, USA] with baseline range from 0.99 to 1.01 and maximum sum of squares (SOS) for the correlation function fit set to 100 (first measurement) or 300 (second measurement).

2.4. Filament incubation reaction

10 μL of 100 μM TIR_{MyD88} (WT/R217A/W284A) was incubated for negative-stain TEM measurement at 277 K in a refrigerator during 7/1/6 days, respectively. 10 μL of 50/15 μM His-GB1-TIR_{MyD88} (WT/L252P) was incubated for negative-stain TEM measurement at 277 K with refrigerator during 3/1 days, respectively. 10 μL of 100 μM TIR_{MyD88} (WT) was incubated for cryo-EM measurement at 303 K in a cell-incubator during 3 h after the incubation at 277 K with refrigerator during 24 h.

2.5. Negative-stain electron microscopy

5 μL of incubated sample was applied to glow-discharged elastic carbon grids, adsorbed onto the grid surface for 10 min, and the grid was washed with Milli-Q water and then blotted twice, and with 1% (w/v) uranyl acetate and then blotted once. The sample was negative-stained with 1% (w/v) uranyl acetate for 1 min and then blotted and air dried for 1 day or more. The specimen was observed on an H-7650 transmission electron microscope (TEM) [Hitachi, Tokyo, Japan] operating at an accelerating voltage of 80 kV.

2.6. Cryo-electron microscopy²

The 2.4 μ l of incubated sample was applied to a Quantifoil grid R1.2/1.3 [Quantifoil Micro Tools, Thüringen, Germany], and the grid was blotted and plunge-frozen into liquid ethane using a fully automated vitrification device, Vitrobot [Thermo Fisher Scientific, MA, USA]. The specimen was observed at a specimen temperature of around 90 K using an FEI Titan Krios TEM [Thermo Fisher Scientific, MA, USA], equipped with a field-emission electron gun operated at 300 kV. The images were recorded on a 4 k $\times$ 4 k 14 μ m-per-pixel direct electron detection camera, Falcon II [Thermo Fisher Scientific, MA, USA]. And the images were collected with the nominal magnification of $\times 75,000$ and the pixel size of 1.1 Å per pixel.

2.7. Image analysis³

Beam-induced motions in the images were corrected by using MOTIONCOR2⁴. Defocus and astigmatism in the image were determined by using Gctf⁵. Images were segmented into 301,207 segment images of 400 $\times$ 400 pixels with a step size of 65 Å along the helical axis with a previously obtained 2D model by using RELION 3.0-beta⁶. The 2D classification, the 3D classification, and the 3D auto-refinement were done by using RELION 3.0-beta. Good class averages in 2D classification, containing 55,127 segment images, were selected for 3D classification. Initial 3D models for 3D classification were created as assumed the circular symmetric bilayer structure in the range from C₂₆ to C₂₈ symmetry and from 240 Å to 280 Å of an outer diameter by using SPIDER⁷. Good class averages with the assumption of C₂₆ symmetry and outer diameter: 260 Å in 3D classification, containing 35,489 segment images, were selected for 3D auto-refinement.

3. Result

3.1 DLS (Dynamic Light Scattering)

Based on the result of HS-AFM, TIR_{MyD88} tends to assemble into oligomers. Thus, we evaluated self-oligomerization of TIR_{MyD88} by using DLS. The first measurement performed immediately after sample filtration showed that the estimated radius of particles was only around 1.5 ~ 2.0 nm (Fig. 1a). Considering the radius of monomeric TIR_{MyD88} (approximately 1.5 nm), this measurement detected only monomeric TIR_{MyD88}. The second measurement after incubation at 277 K for 1 day indicated the presence of larger complexes, whose radius showed distributions around 15 nm and 70 nm (Fig. 1b). This wide distribution pattern suggested that TIR_{MyD88} assembled into the annular complex in solution even without adsorption on mica surface as observed in the previous section, and/or larger complexes like fibril-complex of TIR_{Mal}⁸. Since a signal intensity of DLS is proportional to the molecular weight of the measured sample⁹, population of the large complexes was very small in the measured system.

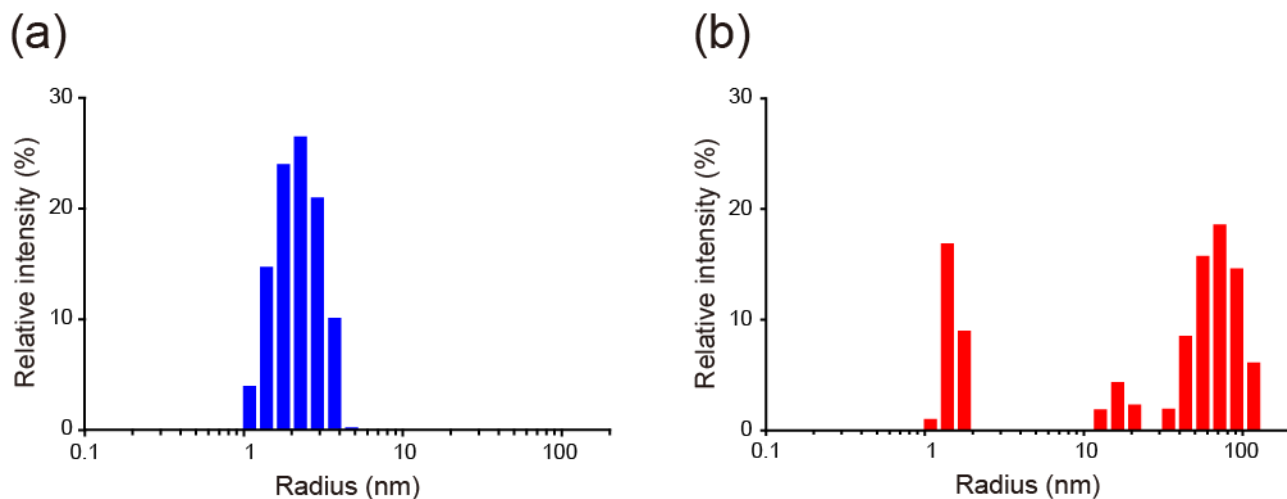


Figure 1 Histograms of DLS-derived radius of gyration of the particles of TIR_{MyD88} before and after 1 day incubation.

The relative intensity histogram of the first measurement (a) and the second measurement (b).

3.2 Negative-stain EM measurement

Since the results of DLS suggested that TIR_{MyD88} could assemble into large complexes even without mica substrate adsorption, we examined TIR_{MyD88} samples incubated at 277K by using negative-stain EM. Fig.2a showed that TIR_{MyD88} (WT) assembled into skewed annular complexes and long tubular-shaped fiber-like complexes similar to the fibril-complex of TIR_{Mal} even without the presence of TIR_{Mal}⁸. I hypothesized that it was a polymer constituted by stacking multiple rings, because the width of the fibrous oligomer was similar to the diameter of the ring and the fibrous oligomer had a striped pattern perpendicular to the long axis of the fibrous oligomer. To further characterize the fibrous oligomer, we created derivatives of TIR_{MyD88} and tested whether they could assemble into fibrous oligomers. TIR_{MyD88} (R217A), which was shown to lose affinity for DD_{MyD88}, could assemble into fibrous oligomers (Fig. 2b)¹⁰. It implied that the intramolecular interaction interface on TIR_{MyD88} did not contribute to forming the fibrous oligomer and that full-length MyD88, which formed the intramolecular interaction, might be able to assemble into the fibrous oligomer. However, TIR_{MyD88} (W284A) did not form any fibrous oligomers but did form many small aggregates (Fig. 2c). This result demonstrated that W284A mutation inhibited the assembly of both the annular complex and the fibrous oligomer.

Notably, TIR_{MyD88} (WT) with His-GB1-tag at the N-terminal did not assemble into any fibrous oligomers but formed numerous ring structures (Fig. 2d). It implied that presence of N-terminal extension of TIR_{MyD88} inhibited ring-stacking in the assembly process of fibrous oligomers, while it did not inhibit ring formation. On the other hand, His-GB1-tag fusion TIR_{MyD88} (L252P) formed huge fibrils constituted from multiple filament-like complexes (Fig. 2e, f). These results demonstrated that L252P mutation promoted assembly of fibril abnormally, and this abnormal fibril assembly might induce pathologic activation of downstream signaling proteins to cause lymphoma¹¹.

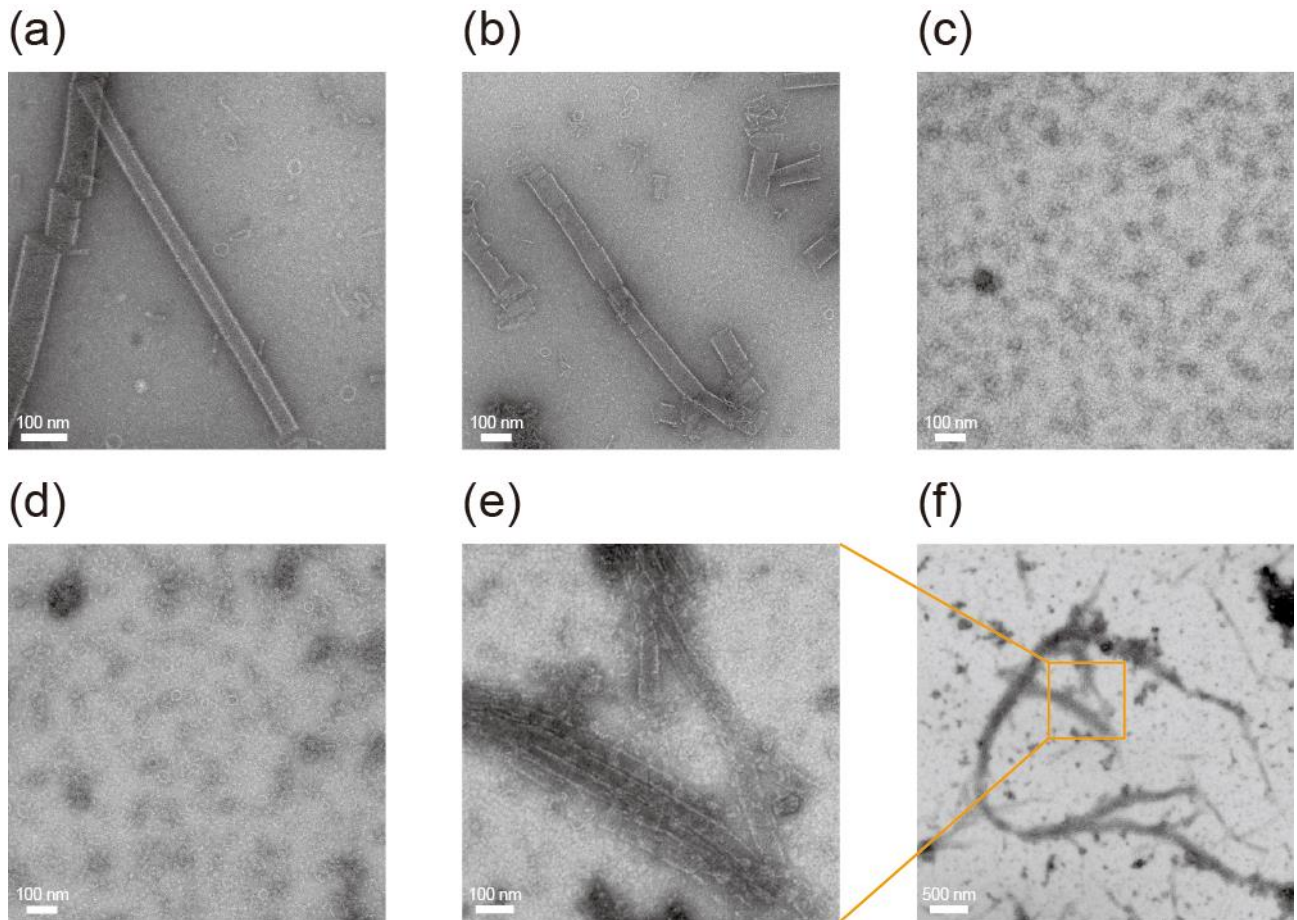


Figure 2 Negative-stain EM images of Filaments.

(a-d) An EM image of (a) TIR_{MyD88} (WT)-fibrous oligomers, (b) TIR_{MyD88} (R217A)-fibrous oligomers. (c) TIR_{MyD88} (W284A)-particles. (d) His-GB1-TIR_{MyD88} (WT)-rings. (e) Magnified EM image of (f). (f) An EM image of fibril aggregation constituted from His-GB1-TIR_{MyD88} (L252P)

3.3 Cryo-EM measurement

Before cryo-EM measurement, we changed the incubation protocol from long incubation at low temperature (277 K) to short incubation at high temperature (303 K), to promote assembly of the fibrous oligomer (Fig. 3a). We observed fibrous oligomers by using cryo-EM with a direct electron detector (Fig. 3b, c). We could not obtain the molecular structure of the fibrous oligomer at atomic resolution, but the image analysis revealed some characteristic features of the fibrous oligomer.

Firstly, 2D class averages of collected images indicated that the fibrous oligomer was constructed from multitude of bilayer structural units (Fig. 4a). The thickness and diameter of the bilayer structural unit were approximately 6.5 nm and 26 nm, respectively. They approximately agreed with the height and diameter of the ring observed in the HS-AFM study, suggesting that multiple of bilayer rings assemble tandemly into the fibrous oligomer.

Secondly, a tentative 3D map was obtained from 3D reconstruction for one bilayer unit of the fibrous oligomer, demonstrating that 26 nodular columns were arranged in a circle at even intervals, providing the structure with 26-fold rotational symmetry (Fig. 4b, c). Although the 26-fold symmetry is presumably correct, quality of the map was insufficient to build a reliable atomic model of TIR_{MyD88} in the fibrous oligomer.

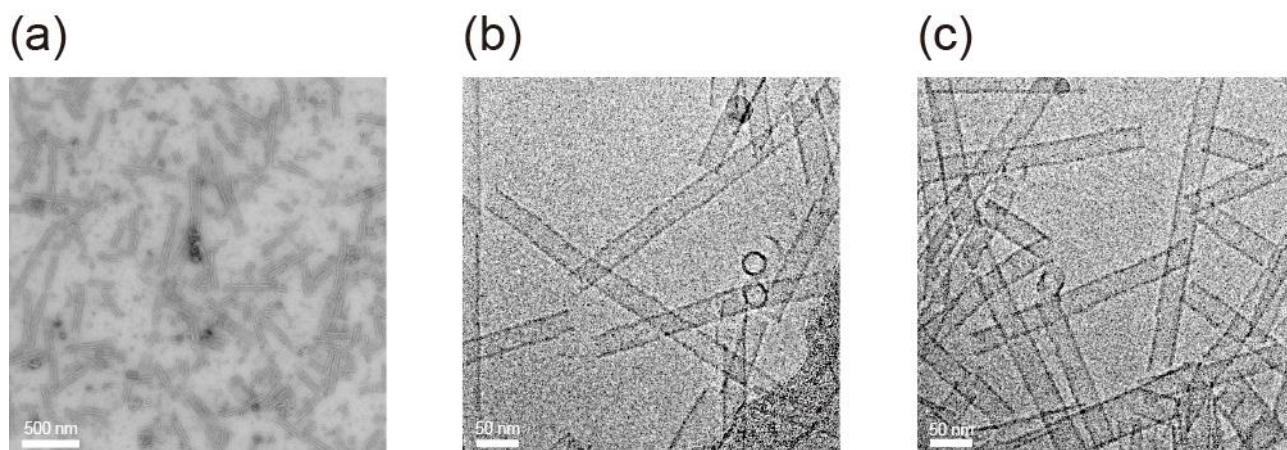


Figure 3 Cryo-EM images of fibrous oligomers of TIR_{MyD88}.

(a) A negative-stained EM image containing numerous TIR_{MyD88} (WT)-fibrous oligomers. (b) A cryo-EM image showing objects imaged from the axial direction where TIR_{MyD88} (WT)-fibrous oligomers look like rings. (c) A cryo-EM image containing numerous TIR_{MyD88} (WT)-fibrous oligomers.

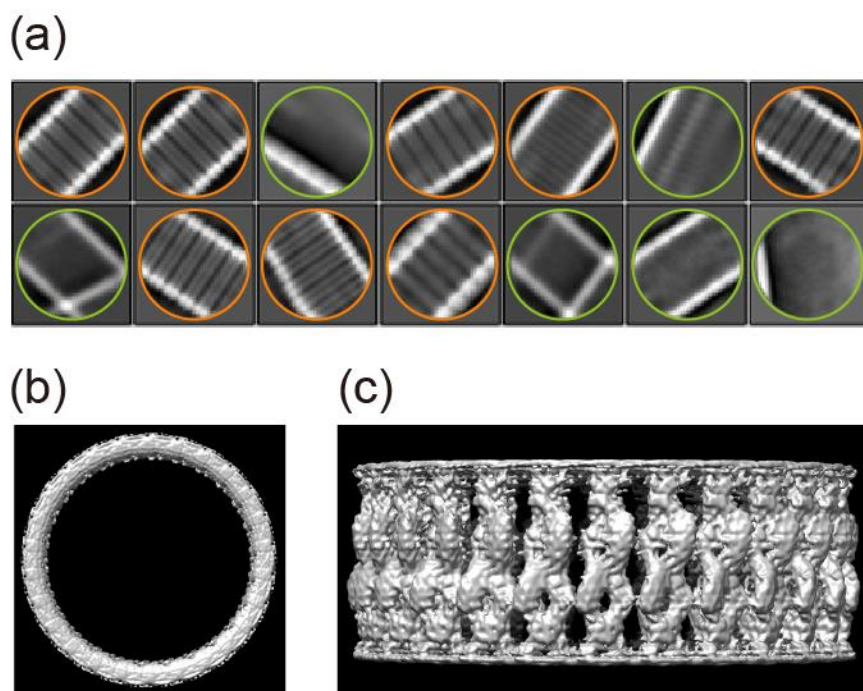


Figure 4 Cryo-EM analysis of the fibrous oligomer.

(a) Representative results of 2D classification. Orange and yellow-green circles correspond to images selected and discarded for further 3D reconstruction procedure, respectively. Each image shows an area of $440 \times 440 \text{ \AA}^2$. Top view (b) and side view (c) of a tentative 3D map of the 26-fold rotational symmetric bilayer unit. Height and diameter of the model were approximately 10 and 28 nm, respectively.

4. Discussion

4.1 Interaction sites in the fibrous oligomer assembly

We observed the fibrous oligomer with cryo-EM but could not reveal its molecular structure in atomic resolution. Nevertheless, we could estimate interaction sites in the oligomer from the results.

Based on the outline of the bilayer ring, it would be reasonable that two interaction sites are essential to maintain the structure, which are orthogonally placed as shown in Figure 5a and 5b. To further clarify this point, I defined two orthogonal directions in the bilayer ring; the lateral and vertical directions, which are along the radial direction and the cylindrical axis of the ring, respectively (Fig. 5a, b). The presence of N-terminal His-GB1-tag abolished the fibrous oligomer assembly, while the ring formation was maintained (Fig. 2d). This result showed the lateral interactions forming the ring is likely independent of the fibrous oligomer assembly.

On the other hand, a single mutation, TIR_{MyD88} (W284A), abolished both the fibrous oligomer assembly and the ring formation (Fig. 2c). Importantly, any filamentous structures were not observed, showing that disruption of the ring can also cause disruption of the fibrous oligomer. Hence, the ring formation may be the primary event, and multiple of rings stack tandemly to form the fibrous oligomer. A previous research proposed a model structure of the TIR_{MyD88}–TIR_{MyD88} oligomer, in which W284 interacted with BB-loop in a head-to-tail style¹². Taken together, W284 would be at the lateral interaction sites forming the ring, while the N-terminal region would be important for the vertical interactions to stack the rings. As the lateral interactions in the ring should be homogeneous, the arrangement of TIR_{MyD88} molecules in the ring would be in a head-to-tail manner. Based on the proposed model of the fibrous oligomer, I defined interactions that form the ring-annular layer, the ring-bilayer, and the bilayer ring stacking as lateral interaction, intra-ring vertical interaction, inter-ring vertical interaction, respectively (Fig. 5c).

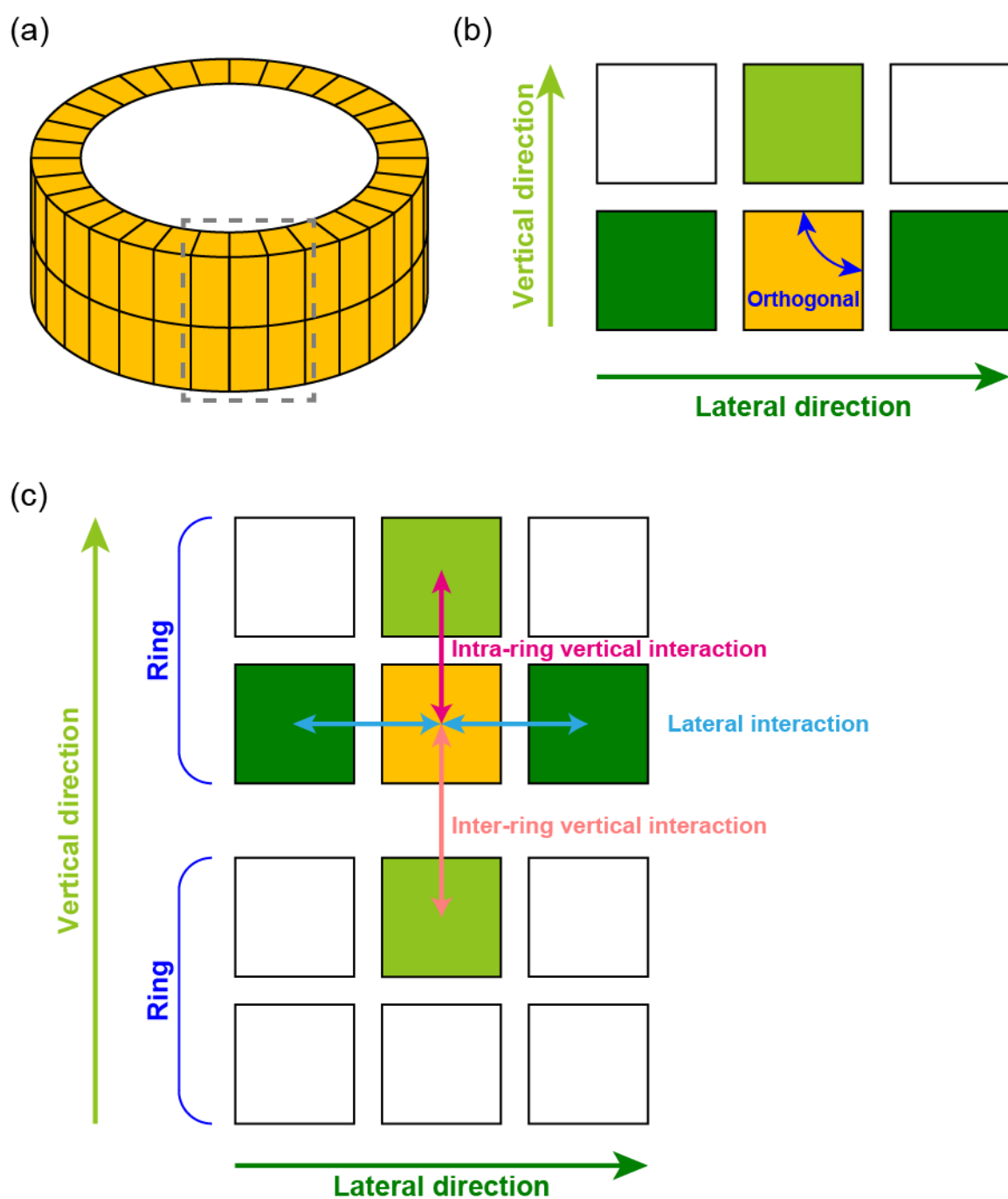


Figure 5 Molecular arrangement in the bilayer ring and the fibrous oligomer.

(a) The schematic structure of the bilayer ring. (b) Array of six monomers enclosed by the gray dotted line in (a). The monomer in orange interacts with the one in yellow-green via the vertical interaction, while with two monomers in green based on the lateral interaction. (c) Array of monomers in the fibrous oligomer. The orange monomer is at the origin and lateral-interactions (cyan), intra-ring vertical interaction (magenta), and inter-ring interaction (pink) are shown.

4.2 Pathogenic mutations of *TIR_{MyD88}*

Previous reports demonstrated that mutations in *TIR_{MyD88}* caused certain types of lymphoma^{13–16}. Reported oncogenic mutations¹¹ include V204F, W205R, S206C, I207T, S209R, M219T, S230N, L252P, and T281P (Fig.6)¹⁷. Since V204, W205, I207, S206, I207, and S209 are at BB-loop that is essential for *TIR_{MyD88}* to function¹⁷, their mutations may disrupt the activity of *TIR_{MyD88}* (Fig. 6). *TIR_{MyD88}* (L252P) was known to have an extraordinarily strong tendency to assemble into fibril complexes, thus it might cause lymphoma via spontaneous formation of Myddosome that leads to constitutive activation of NF- κ B (Fig. 2e, f)¹¹. Because M218 is adjacent to L252P in *TIR_{MyD88}* molecule, M218T might cause a similar phenomenon to L252P (Fig. 6). Moreover, since M218 and L252 locate just inside the intramolecular interaction site with *DD_{MyD88}*, M218T and L252P might weaken auto-suppression for *DD_{MyD88}* and induce Myddosome assembly¹⁰.

On the other hand, there was no clue to infer disease mechanisms of S230N and T281P. However, by looking at the fibrous oligomer of *TIR_{MyD88}*, a putative functional site can be identified on *TIR_{MyD88}*. Since W284 was essential for forming the ring and the fibrous oligomer, W284R mutation may attenuate NF- κ B signaling¹². As T281 locates near W284, T281P might also affect the oligomerization and induce pathological signaling (Fig. 6). Although we have no data about S230 in the complex assembly, S230N may contribute to the intra- or inter-ring vertical interaction (Fig.5c). This is because if W284 is in the lateral interaction, S230 would locate at the sites for the vertical interactions (Fig.6). Moreover, S230 is in the opposite side of N-terminal of *TIR_{MyD88}*, which was critical for the inter-ring vertical interaction. Therefore, we speculate that S230 contribute to the intra-ring vertical interaction.

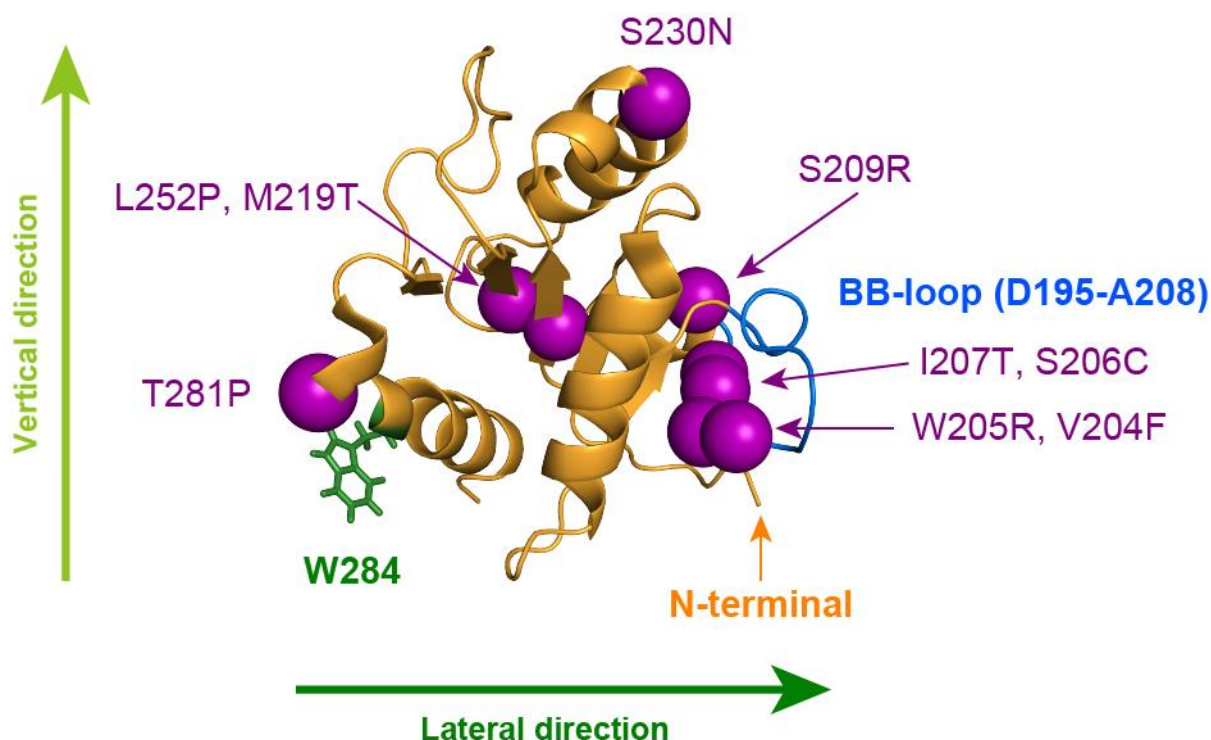


Figure 6 Location of mutated residues in TIR_{MyD88} molecule^{11,17}.

W284, BB-loop, and pathogenic mutations are shown as a green stick, blue loop, and purple sphere, respectively.

4.3 Affinity comparison between homo-interactions of TIR_{MyD88}

We evaluated the strength of affinity of three type interactions that constitute the fibrous oligomer (Fig.5c). HS-AFM observations under tapping force-applied conditions demonstrated many rings, but we could not find the fibrous oligomer. The tapping force derived from HS-AFM observation seems to have destroyed the fibrous oligomer structure, but the bilayer ring structure remained. This difference suggested that the inter-ring vertical interaction in the fibrous oligomer was the weakest affinity in the three interactions (Fig. 5c). At the moment, we did not have any clues to compare strength of the remaining two interactions.

4.4 Predicted molecular mechanism of TLR-Myddosome complexes formation

In full-length MyD88, DD_{MyD88} exists at N-terminal of TIR_{MyD88}. Since presence of His-GB1 at N-terminal of TIR_{MyD88} abolished ring-stacking in the fibrous oligomer assembly process, it might be difficult for full-length MyD88 to assemble into the fibrous oligomer. Moreover, because the ring formation could well

support Myddosome assembly and the affinity of inter-ring vertical interaction was weaker than interactions forming ring, the fibrous oligomer assembly might not be essential. Therefore, we thought that the molecular mechanism of TLR-Myddosome complex centered in MyD88 was based on the ring formation.

5. Conclusion

In this study, we demonstrated that TIR_{MyD88} can form the fibrous oligomer, the long filament-like complex, by using cryo-EM. The resolution of the obtained 3D map was not excellent. Nevertheless, 2D class averages demonstrated that the fibrous oligomer was constituted from multitude of the bilayer ring units. The shape of the bilayer ring well agreed with the ring structure obtained with HS-AFM. Thus, the fibrous oligomer is likely constituted by stacking a number of rings tandemly. Although the fibrous oligomer itself will not be maintained in cells, the homotypic interactions to form the superstructure should be effective and contribute to the formation of MyD88 oligomers observed in cells to form Myddosome. Further cryo-EM study will eventually allow molecular modeling of the fibrous oligomer at atomic resolution. Then, full details of the homotypic interactions of TIR_{MyD88} will be clarified.

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Chapter IV

Molecular analysis of inactivation mechanism of TRPC6 by Calmodulin

Abstract

TRPC6 channels are suppressed by intracellular Ca^{2+} through the negative feedback process known as CDI (Ca^{2+} -dependent inactivation), in which CaM (calmodulin) has been proposed to play a critical role. Our NMR and ITC analyses reveal equal contributions of N- and C-lobes of CaM to initiate CDI, and the stoichiometry of Ca^{2+} -dependent CaM binding to $\text{CBD}_{\text{TRPC6}}$ (TRPC6 CaM-binding domain) is a 1 to 2 ratio. Furthermore, the coiled-coil formation of TRPC6 supported the 1:2 CaM- $\text{CBD}_{\text{TRPC6}}$ binding. This binding mode based on 1:2 stoichiometric ratio provided a novel molecular insight for CDI of TRPC6.

1. Introduction

The Ca^{2+} (calcium ion) influx through calcium channels initiates a crucial chain of events leading to numerous cellular activities including morphological dynamics¹. However, these excessive ions influx trigger aberrant Ca^{2+} -mediated signals and occasionally cause diseases. In kidney podocytes, alterations in Ca^{2+} signaling have been shown to cause proteinuria, and progressive diseases wherein TRPC (Transient Receptor Potential Canonical channel) have been considered a pivotal molecule to induce progressive chronic kidney disease²⁻⁴. TRPC6 is a tetrameric Ca^{2+} permeable non-selective cation channel, and its inherited mutations are known to be associated with FSGS²⁻⁷. To date, nearly 20 FSGS mutations have been reported in TRPC6 channels, and these mutations often exhibit current amplitude amplification and/or delay of channel inactivation (i.e., gain-of-function phenotype)^{8,9}. How do these mutations in TRPC6 cause such functional phenotypes? Since FSGS-associated mutations are expected to be localized in the cytosolic or intracellular membrane side of TRPC6 channels, a regulatory system via intra-cellular events should exist. In previous reports, TRPC6 and nephrotic protein coupling such as Nephhrin through their cytosolic domains have been shown to regulate the surface expression of TRPC6¹⁰⁻¹². Although this mechanistic explanation can account for the current amplification, it cannot satisfy for the delay of inactivation. Therefore, the mechanism of the gain-of-function in TRPC6 activity has not been solved yet.

TRPCs are activated by the stimulation from PLC (phospholipase C)-coupled receptors¹³⁻¹⁵. Activation of PLC leads to the hydrolysis of $\text{PI}(4,5)\text{P}_2$ (phosphatidylinositol-(4,5)-bisphosphate), which in turn produces DAG (diacylglycerol)¹⁶, the physiological agonist of TRPC3/6/7 channels¹³, and also IP_3 (inositol-triphosphate), the store Ca^{2+} release inducer. Consequently, TRPC channels are exposed to substantial elevations of $[\text{Ca}^{2+}]_i$, which occur through TRPC channels, as well as Ca^{2+} release from internal stores. Ca^{2+} -dependent regulation is the most common regulatory mechanism in ion channels for fine-tuning the Ca^{2+} -influx, including TRPC channels. In the case of TRPC4/5 channels, elevation of $[\text{Ca}^{2+}]_i$ leads to the potentiation of channel activity, whereas it generally inhibits other TRPC channels including TRPC6¹⁷⁻²². This negative regulation is termed as CDI (Ca^{2+} -dependent inactivation) and is a central intrinsic feedback system to prevent overload of Ca^{2+} influx. The classical studies of CDI or inactivation of an open-state in the voltage-gated ion

channels have proposed a clear correlation between current amplitudes and inactivation kinetics^{23,24}. These results imply that the involvement of CDI may be reasonable to explain the phenotypes of FSGS mutations^{2,3,8,11,25,26}.

Nevertheless, compared to the classical Ca^{2+} channels, the exact mechanism of CDI in TRPC6 channels and its physiological impact remains mostly unknown. We initially expected that CDI in TRPC6 channels might be caused by the depletion of $\text{PI}(4,5)\text{P}_2$, because $\text{PI}(4,5)\text{P}_2$ mediated regulation has been proposed in a lot of types of TRP channels^{27,28}. However, in the low Ca^{2+} buffering condition, reduction of $\text{PI}(4,5)\text{P}_2$ was not obvious compared to TRPC6 current inactivation in our preliminary data²⁹. Therefore, rather than the depletion of $\text{PI}(4,5)\text{P}_2$, a more direct Ca^{2+} -mediator, CaM (Calmodulin) could be considered in CDI, as the protein has been shown to interact with $\text{CBD}_{\text{TRPC6}}$ (CaM binding domain of TRPC6)^{21,29,30}. In this study, we attempt to understand how CaM mediates CDI on TRPC6 channel.

2. Material and Method

2.1. Construction of expression vectors

pCDNA3.1 vector expressing Human TRPC6 (accession number NM_004621, TRPC6) was provided by Dr. Thomas Hofmann and muscarinic type 1 receptor (M₁R) was prepared as previously reported²⁹. Mutations in CBD or coiled-coil of TRPC6 were generated by overlap extension polymerase chain reaction (PCR) utilizing mutagenic primers where segments were replaced into the *SacI* and *XbaI* sites of TRPC6 expression vector. A similar mutagenesis strategy was used to generate CaM₁₂₃₄ (four Ca²⁺ binding sites mutated), CaM₁₂, and CaM₃₄ using the rat CaM_{WT} in pCI-neo expression vector as a template²¹. Coding regions of 1-75 a.a. and 76-145 a.a. of CaM_{WT} obtained by PCR were cloned to generate the N-lobe of CaM (CaM_{ΔC}) and the C-lobe of CaM (CaM_{ΔN}) in pCI-neo, respectively. FRET and FSGS constructs are described in Appendix Supplementary Information.

2.2. Protein Purification

Rat CaM protein encoded by pET24b [Novagen, WI, USA]³⁰ was expressed in *E. coli* Tuner (DE3) pLysS [Novagen, WI, USA] upon induction by 1.0 mM IPTG for 4 hours at OD 600 nm of 0.7. For ¹³C-¹⁵N-labeled CaMs, proteins were expressed in the M9 minimal medium containing ¹³C labeled glucose and ¹⁵N labeled ammonium chloride. The soluble fraction of CaM was purified by HiTrap Phenyl HP pre-packed columns [GE Healthcare, IL, USA]. To obtain tryptic fragments of CaM (CaM_{ΔN}, CaM_{ΔC}), the purified CaM was digested by trypsin in the presence of Ca²⁺ and subsequently purified by HPLC as described in previous³¹. Preparation of labeled CBD peptide is described in the supplementary.

Preparation of the labeled CBDp (TRPC6-CBD peptide, TRPC6 853-874 a.a.) is as follows. 10xHis-SUMO fusion CBD was expressed in *E. coli* BL-21 (DE3) [Novagen, WI, USA]. This was cultured with M9 Minimal Medium including ¹³C labeled glucose and ¹⁵N labeled ammonium chloride upon induction by 0.2 mM IPTG for 18 hours at induction OD 600nm of 1.0. Cell pellets were initially resuspended in lysis buffer. Resuspended pellets were crushed for 10 min with a sonicator and cell debris were collected following centrifugation. Collected debris were washed by resuspension and centrifugation in lysis buffer and wash buffer

containing 1% Triton-X. Washed debris were resuspended and denatured in denaturation buffer containing 6 M Gdn-HCl. Denatured debris were removed following centrifugation and filtration. Filtered denatured liquid was purified by Ni-NTA resin Purification Column [Roche, Basel, Switzerland]. Eluted fractions concentrated with Amicon Ultra-15 3,000 nominal molecular weight limit (NMWL) [Merck Millipore, MO, USA] to 2 mL and, for refolding, rapidly diluted to 10 times volume in refolding buffer containing 400 mM Arg-HCl. Refolded 10xHis-SUMO3 tag fusion CBD eliminated 10xHis-SUMO3 tag by SENP2. Tag eliminated CBD was concentrated and buffer exchanged to lysis buffer with Amicon Ultra-15 3,000 NMWL. Concentrated CBD was purified by Presep-C C18 (1 mL) [Wako, Osaka, Japan] column. Solvent of eluted fractions were removed with evaporator and freeze dryer [EYELA, Tokyo, Japan]. Dried sample was resuspended in 1% TFA and purified ODS-A column (16 mL) [YMC, Kyoto, Japan]. Fractions of CBD were eluted by 80% acetonitrile gradient. Solvent of eluted fractions were removed with evaporator and freeze dryer. CBD peptide purity was analyzed in 15 % SDS-PAGE and CBD powder was kept in -80 °C.

2.3. NMR titration

NMR measurements were performed at 25, 30 or 37 °C on Bruker AVANCE II and III spectrometers equipped with cryogenic probes (500, 700, and 800 MHz) [Bruker, MA, USA]. All NMR data were processed with NMRPipe software³² and analyzed using Sparky assignment program³³. The NMR samples were prepared in 20 mM Bis-Tris (pH 6.8), 100 mM NaCl, 2-5 mM CaCl₂ and 5-10% D₂O. For the titration experiments, non-labeled CBDp (CBD_{TRPC6} peptide) [TORAY Res. Center, Tokyo, Japan] was added to ¹³C-¹⁵N-labeled CaM (all 200 μM) up to 4.0 molar equivalent (eq), and for CaM_{ΔN} or CaM_{ΔC} up to 2.0 eq. At each titration point, 2D ¹H-¹⁵N SOFAST-HMQC spectra were recorded. The $\Delta\delta$ was calculated according to the formula for each amino acid residue: $\Delta\delta = \sqrt{((\Delta\delta_H)^2 + (\Delta\delta_N/5)^2)}$, where $\Delta\delta_H$ and $\Delta\delta_N$ are the amide proton and nitrogen chemical shift differences, respectively. For assigning the chemical shifts of the backbone nuclei of ¹³C-¹⁵N-labeled CaM, 3D HNCA, HNCACB, HN(CO)CA, CBCA(CO)NH spectra were recorded without or with 2.0 eq of non-labeled CBDp. Cross-peaks in the 2D spectra of other titration points were assigned based on these data with tracing of the chemical shift perturbation. For the assignments of ¹³C-¹⁵N-labeled CBDp, 3D HNCA, HNCACB,

HN(CO)CA and CBCA(CO)NH spectra were recorded with or without non-labeled CaM. The chemical shift deviations from the reference values were used to assess the secondary structure propensities within the assigned residues³⁴.

2.4. ITC (Isothermal Titration Calorimetry)

ITC experiments were performed on the ITC200 [Malvern, Worcs, UK]. The CaM, CaM_{ΔN}, CaM_{ΔC}, and CBDp were dissolved in 20 mM Bis-Tris (pH 6.8), 100 mM NaCl and 2 mM CaCl₂. The CBDp concentration was adjusted to 1.0 mM and titrated into the cell containing CaM at 50 μM or the dissolving buffer and held at 25 °C. The data were obtained from 25 sequential injections of 0.4 μl (initial point), 1 μl (second~17th), or 2 μl (18th~25th) aliquots of the CBDp solution. ITC data were processed in Origin [OriginLab, MA, USA] after subtraction of the background heats of peptide mixing to buffer alone from heats obtained from mixing with CaM. Thermodynamic parameters of CaM solutions were obtained by fitting the data to the independent two binding sites model [Malvern, Worcs, UK], while those of CaM_{ΔN} and CaM_{ΔC} were obtained by fitting the data to the one binding site model.

3. Result

3.1. NMR titration reveals the bidentate interaction between CaM and CBD_{TRPC6}

The structure of the CaM-CBD_{TRPC6} complex in the presence of Ca²⁺ is key to understanding the mechanism underlying CDI. To characterize the complex structure formed by CaM and CBD, we performed NMR titration experiments, which can reveal residue-based structural information, including the binding stoichiometry under Ca²⁺ presence. Upon titrating ¹⁵N-CaM with up to 1 eq of CBDp, the cross-peaks shifted linearly in one direction (Fig. 1a). However, some peaks that were assigned to residues within the N-lobe (1-75 a.a.) of CaM altered the directions with further addition of CBDp (Fig. 1a, Fig. 2a-c). Upon adding more than 2 eq of CBDp, however, no further peak shift was observed (Fig. 2d, e). Based on this observation, the stoichiometry of CaM to CBD_{TRPC6} complex is assumed to be 1:2.

Residues of CaM responsible for interacting with CBDp were identified by the magnitudes of the cross-peak shifts observed between 0 eq and 1 eq ($\Delta\delta_{01}$) and between 1 eq and 2 eq ($\Delta\delta_{12}$), respectively. Most of the cross-peak shifts of the C-lobe residues saturated at $\Delta\delta_{01}$, whereas the cross-peaks of N-lobe residues further shifted by $\Delta\delta_{12}$ (Fig. 2d), indicating that the C-lobe of CaM has a higher affinity for CBD than the N-lobe. The shifts were evident specifically for residues F19, L32, V55, F68, A88, L105, F141, and M145, which have often been identified in the hydrophobic pockets of CaM contributing to target molecule association³⁶. The data suggested that cross-peak shifts, $\Delta\delta_{01}$ and $\Delta\delta_{12}$, were caused by binding through the hydrophobic pockets of the C- and N-lobes, respectively. The binding of CBDp to each lobe was confirmed by additional titration experiments, wherein CBDp was added to ¹³C- and ¹⁵N-labeled CaM_{ΔC} and CaM_{ΔN}, CaM mutants lacking C- or N-lobes, respectively. In both CaM_{ΔC} and CaM_{ΔN}, cross-peak shifts almost saturated upon 1 eq addition of CBDp and there was no directional alternation observed (Figs. 1b, c). These results support the notion that each lobe can bind to a single CBD molecule.

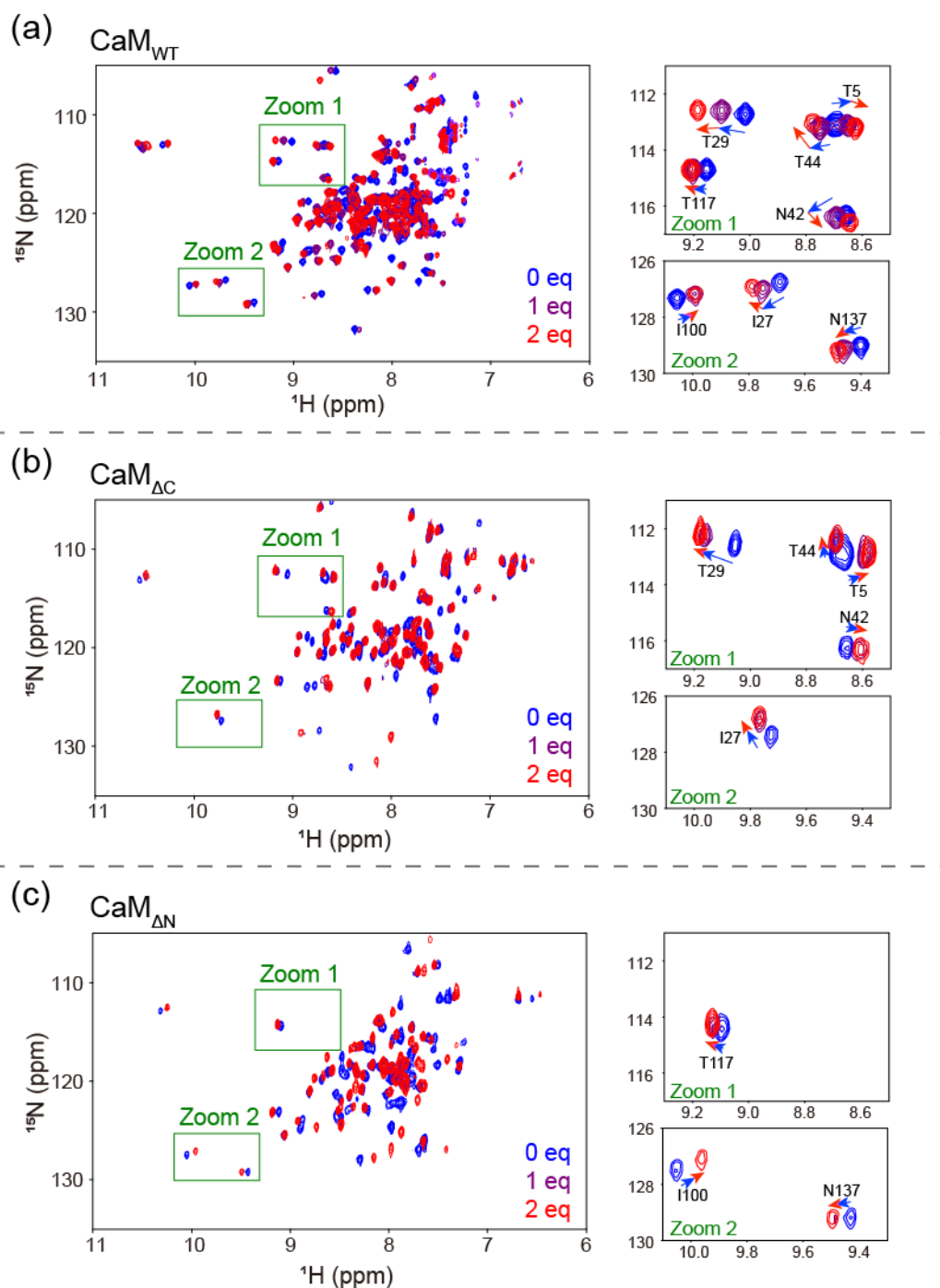


Figure 1 NMR titration of ^{15}N -CaM with CBDp under Ca^{2+} presence.

Overlay of 2D ^1H - ^{15}N SOFAST-HMQC spectra of ^{13}C , ^{15}N -labeled CaM_{WT} (a), $\text{CaM}_{\Delta\text{C}}$ (b), and $\text{CaM}_{\Delta\text{N}}$ (c) with increasing amounts of non-labeled CBDp. The right panels show the zoomed views of cross-peaks. T5, I27, T29, N42, and T44 in the N-lobe of CaM_{WT} showed direction changes in the titration although others shifted linearly.

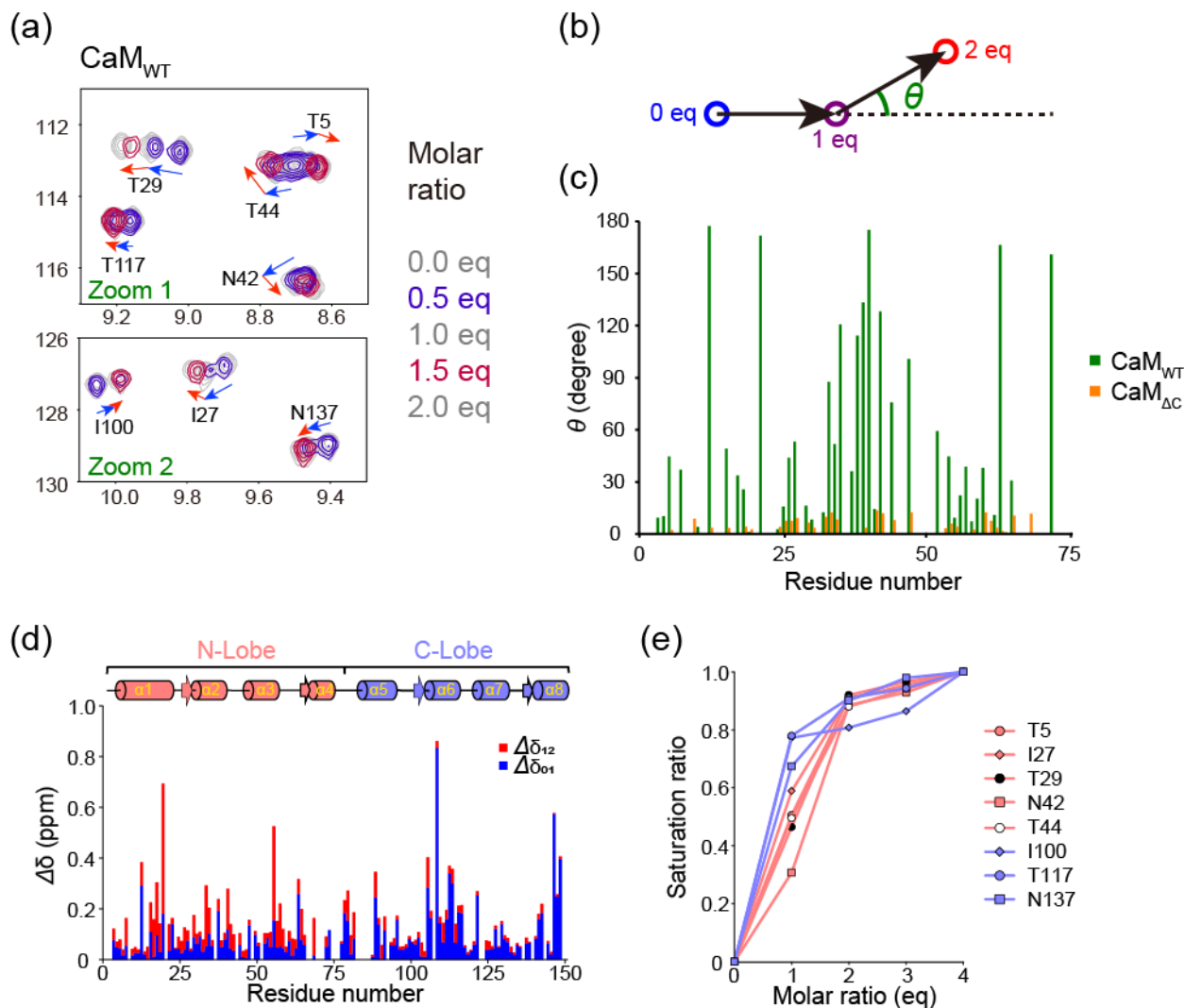


Figure 2 Detailed analysis of NMR titrations of CaM derivatives with CBDp.

(a) The same spectra as in Fig. 1a right but including those recorded with 0.5 (dark blue) and 1.5 eq (dark red) of non-labeled CBDp, showing a switch of the exchange mode from the slow (0 to 1 eq) to fast (1 to 2 eq) regime. (b) The schematic of direction alternation quantified as the angle θ formed by the two vectors shown, which were calculated by the following equation: $\theta = (180/\pi)\arccos(-(\Delta\delta_{01}^2 + \Delta\delta_{12}^2 - \Delta\delta_{02}^2)/2\Delta\delta_{01}\Delta\delta_{12})$. (c) The plot of angle calculated based on data obtained for the N-lobe of CaM_{WT} (Fig. 1a) and $\text{CaM}_{\Delta\text{C}}$ (Fig. 1b). The C-lobe residues were not included as their shifts essentially saturated at 1 eq. (d) Combined chemical shift perturbations (CSPs) upon addition of CBD were plotted as a function of the residue number of CaM. In stacked bar graph, $\Delta\delta_{01}$ (blue) and $\Delta\delta_{12}$ (red) represent CSPs from 0 eq to 1 eq and from 1 eq to 2 eq, respectively. (e) Saturation of chemical shift changes as a function of the equivalent molar ratio of added CBDp to CaM_{WT} . Each value ($\Delta\delta_{ij}$) was divided by $\Delta\delta_{\text{total}}$ ($=\Delta\delta_{01} + \Delta\delta_{12} + \Delta\delta_{23} + \Delta\delta_{34}$) to yield the saturation ratio. All residues reached 80-90% of saturation at 2 eq.

3.2. ITC measurements for determining stoichiometry

The stoichiometries of the binding of CBDp to CaM, N-lobe, or C-lobe were further validated with ITC experiments. Here, again CBDp was added to either CaM_{WT}, CaM_{ΔC} or CaM_{ΔN} under Ca²⁺ presence. The thermogram of CaM_{WT}-CBDp demonstrated a convex exothermic response that was maximized near 1 eq of CBDp, and then diminished approximately at 2 eq (Fig. 3a). This thermogram was fitted with an independent two binding sites model. The site 1, which is responsible for the first half of the reaction, exhibited a very small reaction heat with a high ligand affinity (30 nM, Table 1), whereas the site 2 exhibited greater reaction heat and a lower affinity (4.8 μM) than the site 1. The independent two-sites association was confirmed by using the lobe-separated CaM, where the thermogram of CaM_{ΔC} was a sigmoidal exothermic curve with a low affinity to CBDp (3 μM) (Fig. 3b), demonstrating a similar affinity to the site 2 of the CaM_{WT}-CBD thermogram. Though CaM_{ΔN} exhibited clear binding to CBDp in the NMR titration experiments (Fig. 3b), CaM_{ΔN} in ITC measurement exerted nearly a flat thermogram, in which the thermic reaction was very weak (Fig. 3c). This observation qualitatively agrees with the above result of CaM_{WT} that the reaction heat exerted by site 1 was very small. Accordingly, the 1:2 stoichiometry and independent CBD binding to the C- and N-lobe of CaM, were verified by NMR and ITC experiments.

Table 1 Thermodynamic parameters of the binding of CaM to CBD.

	N	K _d (μM)	ΔH (kcal mol ⁻¹)	-TΔS (kcal mol ⁻¹)	ΔG (kcal mol ⁻¹)
CaM _{WT} (Site 1)	0.532 ± 0.102	0.0313 ± 0.016	-0.887 ± 0.526	-9.42 ± 0.21	-10.3 ± 0.39
CaM _{WT} (Site 2)	0.678 ± 0.045	4.84 ± 1.45	-4.03 ± 0.04	-3.23 ± 0.21	-7.27 ± 0.17
CaM _{ΔC}	0.910 ± 0.004	2.59 ± 0.63	-5.07 ± 0.19	-2.56 ± 0.04	-7.63 ± 0.15
CaM _{ΔN}			N/A		

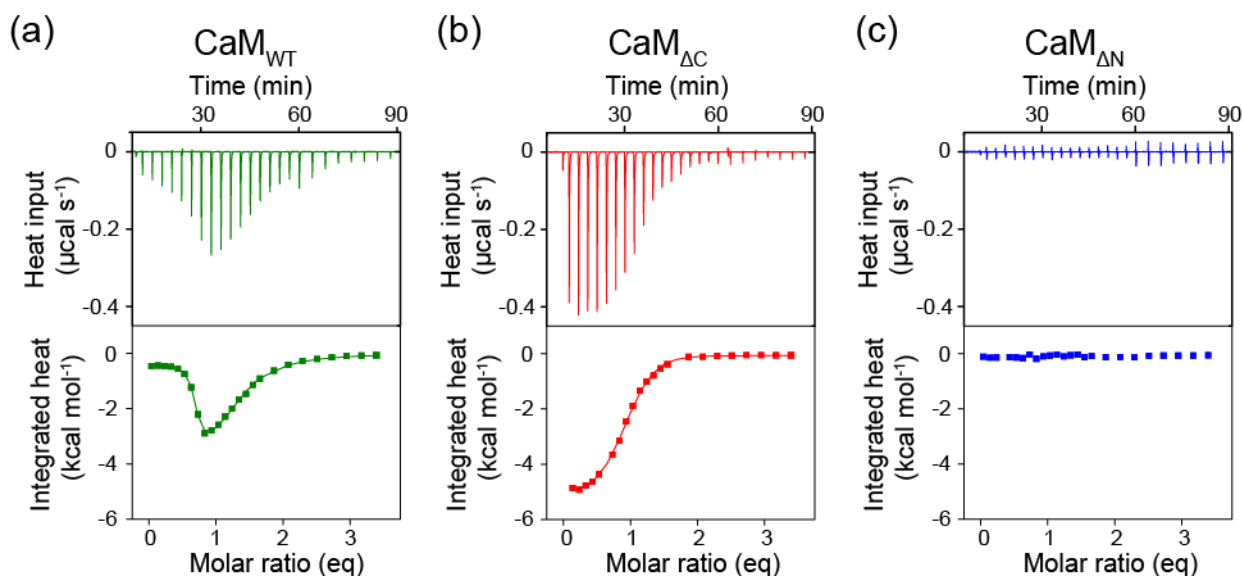


Figure 3 ITC analysis for interactions between CBDp and CaMs under Ca^{2+} presence.

ITC thermograms for CBD binding of CaM_{WT} (a), $\text{CaM}_{\Delta\text{C}}$ (b), and $\text{CaM}_{\Delta\text{N}}$ (c) in the presence of Ca^{2+} . Upper panel/raw data for sequential injections of 1.0 mM of CBDp into 0.05 mM of CaM solutions. Lower panel, the data points obtained by integration of the peaks in the upper panel, and plotted against the molar ratios.

3.3. NMR titration of CBDp with CaM

Upon CaM complex formation, CBDs often adopts an alpha-helical conformation³⁴. To confirm the structural re-arrangement of CBD by CaM binding, we performed NMR titration experiments, in which non-labeled CaM was added to ^{13}C , ^{15}N -labeled CBDp, and 2D ^1H - ^{15}N HMQC spectra were recorded at each titration point under Ca^{2+} presence (Fig. 4a-c). Considering the results of the titration experiment using ^{15}N -CaM at 1 eq (Fig. 1), the major cross-peaks at the 1 eq should correspond to the C-lobe binding state of labeled-CBDp (Fig. 4b). The secondary structure of CBDp can be predicted from the chemical shift values of $^{13}\text{C}_\alpha$ and $^{13}\text{C}_\beta$. In the CaM-free condition, the chemical shifts indicated CBDp adopted random-coil form. However, with Ca^{2+}CaM , the residues in CBD from Y855 to K864 formed an α -helix, but those from R865 to K874 still remained in a random-coil structure (Fig. 4d). The result showed that CaM held a shorter helix (10 amino acid residues) of CBDp, which supported the single lobe binding compared with the other typical longer helix CBD in 1:1 stoichiometry binding proteins.

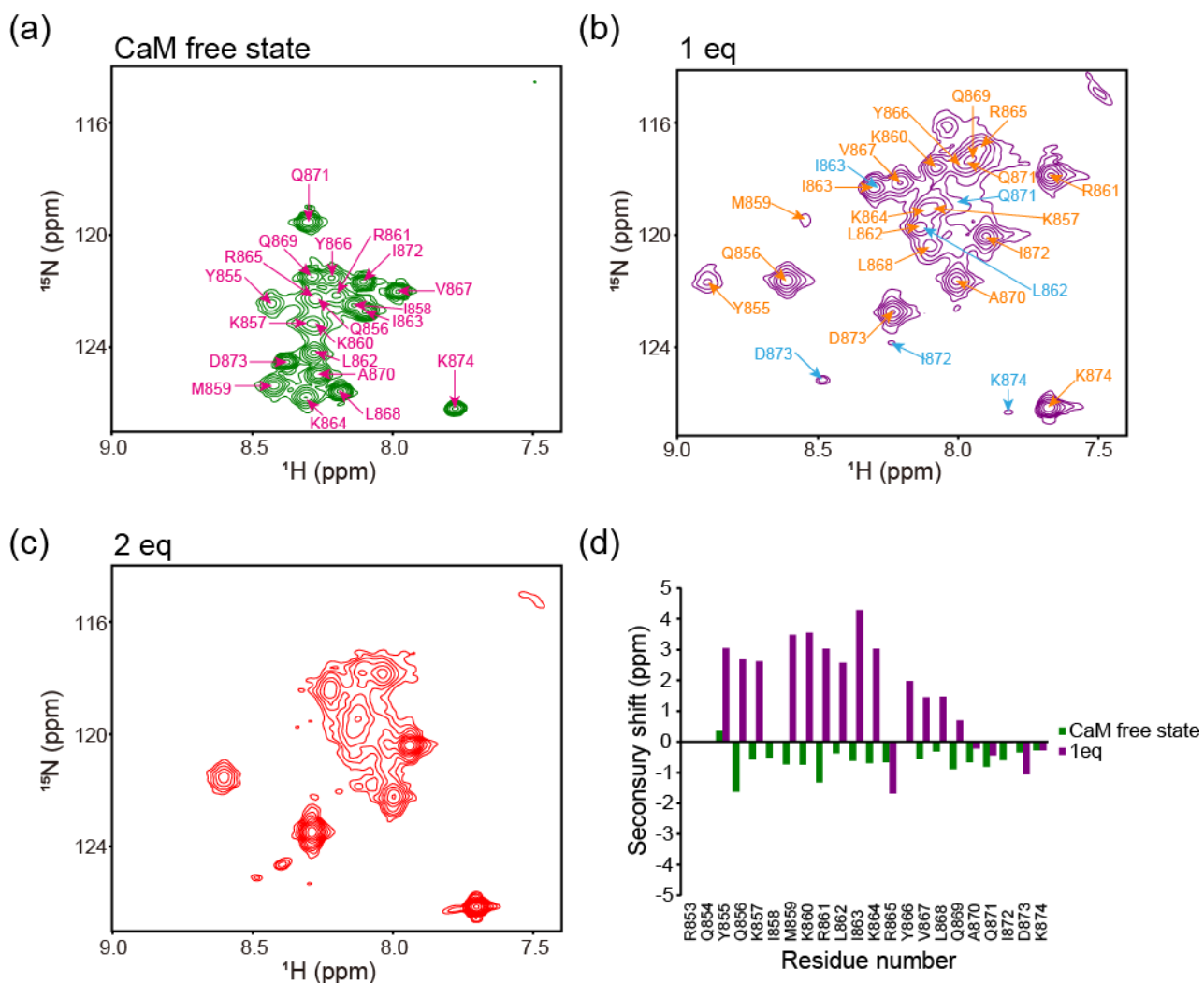


Figure 4 NMR analysis of the binding of ^{13}C , ^{15}N -CBD to CaM.

(a, b) 2D ^1H - ^{15}N SOFAST-HMQC spectra of ^{13}C , ^{15}N -labeled CBD in the absence (green) and the presence of 1 eq CaM (purple). In the 1 eq state, assignments are indicated for the major (orange), and minor state (cyan), which presumably correspond to the C- and N-lobe bound state, respectively. However, most of the signals at the 1:2 ratio of CaM:CBDp state (in the presence of 0.5 eq of CaM, red) (c) were extensively broadened most likely due to intermediate exchange between multiple states (free, the C- and N-lobe bound states) of ^{13}C , ^{15}N -CBD, hence assignment was not obtained. (d) Plots of the secondary chemical shifts of ^{13}C , ^{15}N -CBD obtained in the absence (green) and the presence of 1 eq CaM (purple). The secondary shift was calculated according to the formula: $[\delta^{13}\text{C}_\alpha (\text{measured}) - \delta^{13}\text{C}_\alpha (\text{random coil reference})] - [\delta^{13}\text{C}_\beta (\text{measured}) - \delta^{13}\text{C}_\beta (\text{random coil reference})]$. Small absolute values seen for the CaM free state show that the peptide is in the random coil in solution. However, from Y855 to K864, addition of 1 eq of CaM gave rise to a substantial increase in secondary shift values, indicating the formation of an α -helix in this region.

4. Discussion

4.1. The interaction between CaM and CBD_{TRPC6}

In this study, we uncovered the stoichiometry of binding between CaM and CBD_{TRPC6} is 1:2 under Ca²⁺ presence. The stoichiometry of canonical CaM-CBD binding is 1:1³⁵, in which two lobes (N- and C-lobe) bind to one long α -helix of CBD. This is in contrast to our results, in which each of the N- and C-lobe binds one CBDp. The affinity of the C-lobe binding to CBD is 100-fold higher than that of the N-lobe binding (Fig. 3 and Table 1). The relatively short α -helical structure formed by CBDp of TRPC6 for binding CaM (Fig. 4) may present a crucial structural constraint for the 1:2 stoichiometry. More specifically, while longer α -helix CBDs in classical CaM target proteins (the length is often more than 15 amino acid residues) allow simultaneous contact from both lobes of CaM thus resulting in a 1:1 ratio, a shorter α -helical CBD may well-fit into a single-lobe binding. Indeed, such a non-canonical stoichiometry has been reported for a few CaM binding proteins^{34,36–38}, in which the CBDs folded into short α -helical structures.

4.2. The contribution of coiled-coil to the function of TRPC6

The ‘coiled-coil’ in TRP channels has multiple functions such as channel tetramerization, surface trafficking, assembly to another molecule^{39–42}, and even in channel gating regulation⁴³. In TRPC6, the coiled-coil region exists next to CBD. Thus, formation of the coiled-coil bundle may bring multiple CBDs in close proximity, promoting the 1:2 binding of CaM-CBD_{TRPC6}. In fact, my collaborators demonstrated that deletion of the coiled-coil segment severely impaired CDI, probably because two CBDs were not in close proximity, abolishing the 1:2 CaM-CBD binding (unpublished data). Therefore, the coiled-coil segment of TRPC6 likely has a fundamental role in producing the CaM-mediated CDI. Structurally, the coiled-coil could support the 1:2 CaM-CBD binding, which acts as a ‘bridge’ between the two subunits of a tetrameric TRPC6 complex, thus potentially acting as a minimal unit for inactivation.

4.3. Molecular structure of full-length TRPC6

During this paper writing, the cryo-EM of TRPC6 structure in inactivated state has been published from two groups, in which both structures showed a parallel tetrameric coiled-coil bundle (Fig.5)^{44,45}. However, it is curious that CBD_{TRPC6} (in the papers described as a horizontal helix) is almost concealed by the N-terminal cytoplasmic region including ankyrin domain. For CaM binding, this N-terminal region will need to dissociate from CBD_{TRPC6}. Previous works on TRPC3 and temperature sensitive TRP (TRPV4) demonstrated that the N-terminal region of TRP channels interacts with membrane components such as inositol lipids 'PI(4,5)P₂'⁴⁶⁻⁴⁸. These observations imply that CBD can be uncovered in cellular environments. Therefore, although CaMs might be able to access adjacent CBD_{TRPC6} in the activated TRPC6 tetramer, how to inactivate TRPC6 after formation of two bridging interactions between CaM and CBD_{TRPC6} has not been revealed.

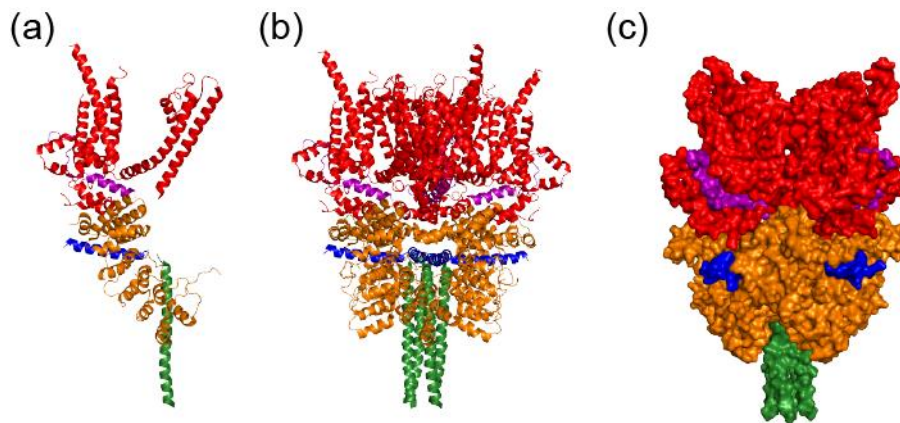


Figure 5 Molecular structure of inactivated TRPC6⁴⁵.

(a) The structure of TRPC6 monomer drawn in ribbon model. Red, purple, orange, blue, and green parts correspond to transmembrane domain, TRP box domain, Ankyrin domain, CaM binding domain, and coiled-coil domain, respectively. (b) The structure of TRPC6 tetramer drawn in ribbon model. (c) The structure of TRPC6 tetramer drawn in surface model. CBD_{TRPC6} (blue) is mostly covered by Ankyrin domain (orange).

5. Conclusion

In conclusion, the present study revealed the binding mode of CaM-CBD_{TRPC6}, which provided a novel molecular insight for Ca²⁺-mediated negative gating regulation of TRPC6 channel.

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Chapter V

Additional Discussion and Concluding Remarks

1. Novel therapeutic approaches for MyD88-dependent lymphoma based on the molecular function of MyD88

L252P mutation in TIR_{MyD88} is well known as a representative causative mutation for DLBCL (diffused large B-cell lymphoma) and exhibits a gain-of-function phenotype, which indicates constant activation of NF- κ B underlies the pathomechanism¹⁻³. Although causal mutations involved in lymphoma in TIR_{MyD88} were identified, it had been difficult to develop therapeutic approaches, because detailed molecular function of TIR_{MyD88} revealed so far was limited in the peripheral region of BB-loop. In this thesis, I proposed that TIR_{MyD88} functioned as an auto-suppressor for DD_{MyD88} via intramolecular interaction and as a scaffold for DD_{MyD88} oligomerization to assemble Myddosome. The L252P mutation would affect both functions. From the viewpoint of the intramolecular interaction, the L252P mutation may attenuate the affinity of intramolecular interaction that suppresses the homo-oligomerization of DD_{MyD88}, because L252 is located just inside the intramolecular interaction site centered in R217. On the other hand, from TEM images of purified His-GB1-TIR_{MyD88} (L252P), we found that the L252P mutation strongly induced fibril formation, which may substantially promote spontaneous Myddosome assembly. Therefore, we propose that these two outcomes derived from L252P mutation induce the constant activation of NF- κ B via the MyD88-dependent canonical pathway in the lymphoma. Thus, inhibiting DD_{MyD88}-DD_{MyD88} homo-interactions or TIR_{MyD88}-TIR_{MyD88} homo-interactions that underlie the Myddosome formation is the key to resolve the L252P-derived constitutive activation of NF- κ B. And molecules exhibiting such inhibitory effect would become excellent drugs to treat the lymphoma caused by L252P and its related mutation.

2. Relationship between MyD88 and TRPC6

Although the immune system is constituted from immunocytes, immune proteins, and their functional network, cells and proteins working in other systems can affect the immune system. For instance, the fibroblast-like synoviocyte, which is not classified into immunocytes, takes an important role in the pathogenic mechanism of rheumatoid arthritis that is one of representative auto-immune disease^{5,6}. An enzyme called STEAP4 that functions in the metabolic system contributes to the regulation of inflammation and has some role in the pathogenic mechanism of rheumatoid arthritis^{7,8}.

TRP family channel proteins are also one such protein⁹. For instance, ligand recognition of TLR4 and NOD2 induced the activation of TRPC6 via DAG (diacylglycerol) and Ca^{2+} influx^{10,11}. Besides this, activated TLR4 induced activation of other TRP channels such as TRPV1, TRPV2, and TRPM4 (Fig.1)¹²⁻¹⁴. Since TLR4 activation affected multiple TRP channels, Myddosome might contribute to the regulation of these channels. However, details of the molecular mechanism how PRRs including TLR4 affects TRP channels has not yet been revealed, and further studies are necessary.

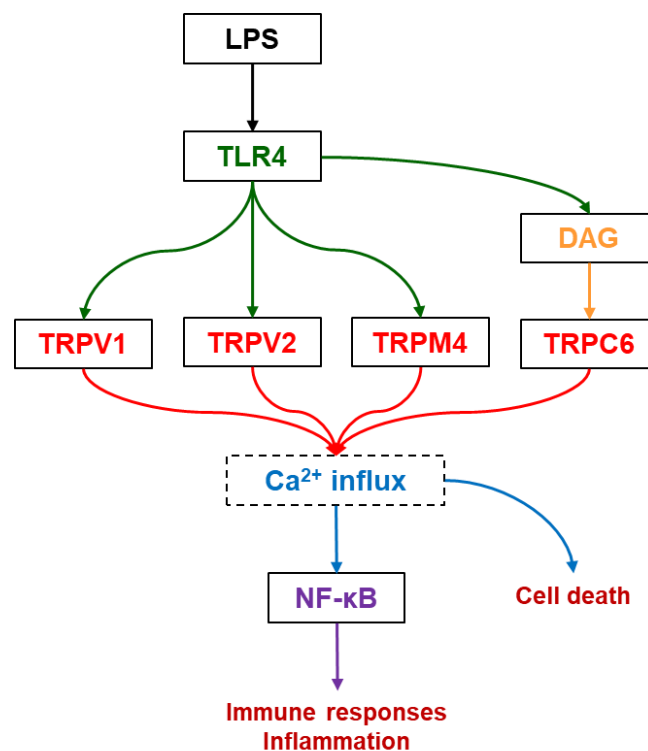


Figure 1 Schematic of speculated signal pathways including TLR4 and TRP channels⁹.

TLR-mediated inflammatory responses might be mediated by Ca^{2+} influx through TRP channels.

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Concluding Remarks

In this thesis, I mainly describe the molecular analysis of MyD88 based on various interactions of TIR_{MyD88}. I succeeded the purification of MyD88^{FL} for the first time in the world, and the analysis for the intramolecular interaction between TIR_{MyD88} and DD_{MyD88} proposed an auto-suppressive regulation of MyD88 by blocking the functional site on DD_{MyD88} (Fig.2a). Moreover, we demonstrated that TIR_{MyD88} assembled into annular homo-oligomers (Fig.2b). The interactions underlying the oligomer formation would also underlie the Myddosome formation by supporting oligomerization of DD_{MyD88}. From these results, I propose the multi-steps molecular mechanism from ligand binding of TLR to Myddosome formation (Fig. 2c).

Information obtained throughout this study, such as molecular mechanisms of action of MyD88 as well as locations of its protein-protein interaction sites, constitutes a solid basis for further structural analysis of the receptor-Myddosome complexes. In addition, the data obtained here will contribute to the drug development for the treatment of MyD88-dependent diseases such as diffused large B-cell lymphoma.

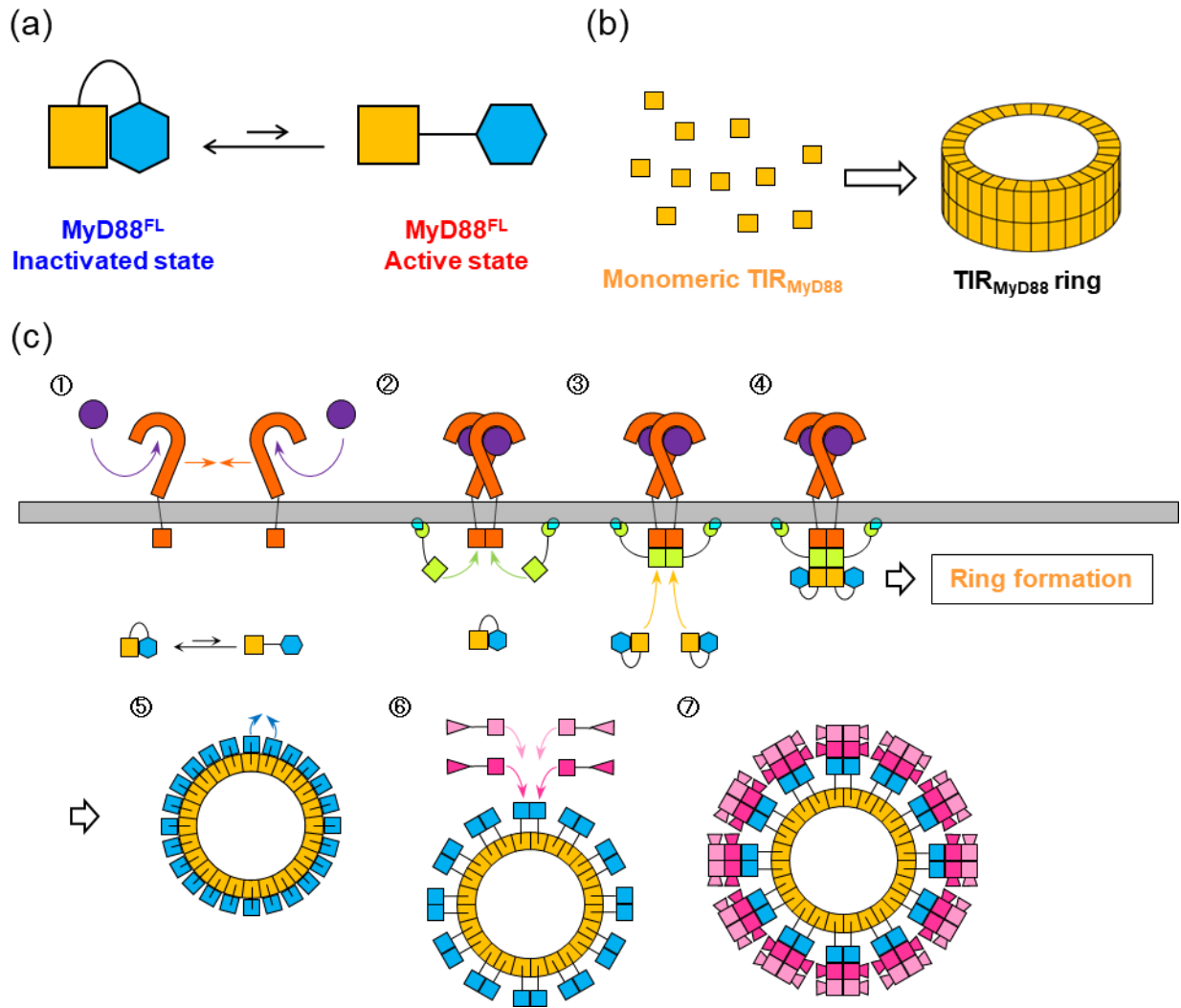


Figure 2 Schematics of the proposed molecular mechanism of MyD88.

(a) The schematic of auto-suppressive model of MyD88^{FL}. (b) The schematic of annular homo-oligomer formation of TIR_{MyD88}. (c) Schematics of the proposed molecular mechanism of Myddosome assembly based on auto-suppressed regulation of MyD88^{FL} and oligomerization of TIR_{MyD88}. (1) The extracellular domain of TLR4 (orange) recognizes a pathogenic ligand molecule (purple) and ligand binding induces dimerization of TLR4. At this situation, MyD88 mostly exists in the auto-suppressed state. (2) The dimerized intracellular domains of TLR4, TIR_{TLR4}, recruit and induce dimerization of TIR_{Mal} (light green). (3) Dimerized TIR_{Mal} recruits MyD88 via interaction between TIR_{Mal} and TIR_{MyD88} (yellow), inducing dimerization of MyD88. (4) Dimerized MyD88 assemble into a homo-oligomer of TIR_{MyD88} to form the ring structure. (5) The ring formation induces DD_{MyD88} (blue) homo-oligomerization by increasing local concentration of MyD88. (6) Homo-oligomers of DD_{MyD88} recruit IRAK4 and IRAK1/2 to assemble into Myddosome around the ring. (7) Multiple Myddosome assemblies around the ring induce auto-phosphorylation of IRAKs, which triggers downstream signaling.

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