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IV. 研究成果の刊行物・別冊

TRAM Is Involved in IL-18 Signaling and Functions as a Sorting Adaptor for MyD88

Hidenori Ohnishi^{1*}, Hidehito Tochio^{2*}, Zenichiro Kato¹, Norio Kawamoto¹, Takeshi Kimura¹, Kazuo Kubota¹, Takahiro Yamamoto¹, Tatsuyoshi Funasaka³, Hiroshi Nakano³, Richard W. Wong³, Masahiro Shirakawa², Naomi Kondo¹

1 Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan, **2** Department of Molecular Engineering, Graduate School of Engineering, Kyoto University, Kyoto, Japan, **3** Laboratory of Molecular and Cellular Biology, Department of Biology, School of Natural System, Kanazawa University, Kakuma-machi, Kanazawa, Japan

Abstract

MyD88, a Toll/interleukin-1 receptor homology (TIR) domain-containing adaptor protein, mediates signals from the Toll-like receptors (TLR) or IL-1/IL-18 receptors to downstream kinases. In MyD88-dependent TLR4 signaling, the function of MyD88 is enhanced by another TIR domain-containing adaptor, Mal/TIRAP, which brings MyD88 to the plasma membrane and promotes its interaction with the cytosolic region of TLR4. Hence, Mal is recognized as the “sorting adaptor” for MyD88. In this study, a direct interaction between MyD88-TIR and another membrane-sorting adaptor, TRAM/TICAM-2, was demonstrated *in vitro*. Cell-based assays including RNA interference experiments and TRAM deficient mice revealed that the interplay between MyD88 and TRAM in cells is important in mediating IL-18 signal transduction. Live cell imaging further demonstrated the co-localized accumulation of MyD88 and TRAM in the membrane regions in HEK293 cells. These findings suggest that TRAM serves as the sorting adaptor for MyD88 in IL-18 signaling, which then facilitates the signal transduction. The binding sites for TRAM are located in the TIR domain of MyD88 and actually overlap with the binding sites for Mal. MyD88, the multifunctional signaling adaptor that works together with most of the TLR members and with the IL-1/IL-18 receptors, can interact with two distinct sorting adaptors, TRAM and Mal, in a conserved manner in a distinct context.

Citation: Ohnishi H, Tochio H, Kato Z, Kawamoto N, Kimura T, et al. (2012) TRAM Is Involved in IL-18 Signaling and Functions as a Sorting Adaptor for MyD88. PLoS ONE 7(6): e38423. doi:10.1371/journal.pone.0038423

Editor: Tianyi Wang, University of Pittsburgh, United States of America

Received: February 8, 2012; **Accepted:** May 9, 2012; **Published:** June 7, 2012

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Funding: This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Sports Science, Culture and Technology of Japan and by Health and Labour Science Research Grants for Research on Intractable Disease from the Ministry of Health, Labour and Welfare, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ohnishih@gifu-u.ac.jp (HO); tochio@moleng.kyoto-u.ac.jp (HT)

Introduction

Toll-like receptors (TLRs) are representative innate immune receptors that recognize pathogen-associated molecular patterns (PAMPs). MyD88, a cytosolic adaptor protein, is involved in the signaling pathways initiated by all of the reported TLRs with the exception of TLR3 [1]. Usually, PAMPs first bind to the extracellular domain of the TLRs, and the cytosolic region of TLRs then interact with MyD88, which allows the signal to be transmitted to the downstream kinase Interleukin (IL)-1 receptor associated kinase 4 (IRAK4). The resulting activation of IRAK4 eventually leads to the activation of the transcription factors NF- κ B and AP-1 via conserved phosphorylation cascades [2]. MyD88 is composed of two functional domains: an N-terminal death domain (DD) and a C-terminal Toll/Interleukin-1 receptor homology (TIR) domain [3]. The DD is a protein interaction module that is involved in a variety of cellular events. Similar to the DD, the TIR domain also mediates protein-protein interactions via homotypic TIR-TIR interactions. In contrast to the DD, the TIR domain is almost exclusively found in the TLR related cytosolic adaptors or in the cytosolic regions of the TLRs, IL-1 and IL-18 receptors. Homotypic interactions of these protein interaction modules play a pivotal role in transmitting the signals

downstream from the TLR; the TIR of MyD88 interacts with the TIR of the TLRs, and the DD of MyD88 interacts with the DD of IRAK4, which forms a large protein complex called the Myddosome [4].

TLR4 signaling, the best characterized signaling pathway among a dozen of known TLR pathways, is activated by lipopolysaccharide (LPS) from gram-negative bacteria, and the pathway plays a major role in endotoxin shock. Two modes of the signaling have been described: the MyD88-dependent and MyD88-independent pathways. In the MyD88-dependent TLR4 signal transduction pathway, another TIR domain-containing adaptor protein, Mal (also called TIRAP), plays an important role. Mal binds MyD88 via a homotypic TIR interaction and then associates with the plasma membrane using its PIP2-binding domain. Thus, Mal has been suggested to serve as a “sorting adaptor” that recruits the “signaling adaptor”, MyD88, to the membrane region where the activated TLR4 resides [5]. Mal is not an essential factor for the signaling because signal transduction can occur even without Mal [6,7], but Mal can substantially facilitate the signaling. A pair of TIR domain-containing adaptors, TIR domain-containing adaptor inducing IFN- β (TRIF) and TRIF-related adaptor molecule (TRAM), is known to play important roles in the MyD88-independent TLR4-signaling

pathway in which TRIF and TRAM function as the signaling and the sorting adaptors, respectively. This pathway transmits signals from TLR4 at early endosomes after the LPS-induced internalization of TLR4 [8,9]. TRAM is known to deliver TRIF to the endosomes via a specific region of the plasma membrane by using its myristoylation site and polybasic region [10]. These findings indicate that the specific combinations of the sorting and the signaling TIR-containing adaptors define the specific signal transduction pathways.

MyD88 is also involved in acquired immune responses because it mediates the signals from the inflammatory cytokines IL-1 and IL-18; ligand-activated IL-1/IL-18 receptors that subsequently interact with MyD88 to trigger downstream protein kinase cascades that eventually activate the transcription factors NF- κ B and AP-1 in a similar manner to TLR signaling. Although the intracellular signaling pathway is similar to the MyD88-dependent TLR4 pathway, the sorting-adaptor Mal is not involved [11]. As mentioned above, TLR4 signaling is facilitated either by Mal (MyD88-dependent pathway) or TRAM (MyD88-independent pathway) in a pathway-dependent manner. The former mainly localizes in PIP2 rich plasma membrane regions, while the latter is found not only in the plasma membrane but also in the internalized early endosomes that dispatch the signals. Thus, different sorting adaptors recruit MyD88 to different membrane regions and create distinct types of signal initiation complexes. In contrast to TLR signals, IL-1/IL-18 signaling has not thus far been thought to require such sorting adaptors. Interestingly, Kagan *et al.* reported that an engineered MyD88 that is endowed with PIP2 binding ability could rescue the LPS-TLR4 signaling in mouse embryonic fibroblast (MEF) cells from MyD88 and Mal double knockout mice, although it failed to rescue IL-1 signaling in the cells [5]. This observation suggests that TLR4 and IL-1R are located in distinct regions of the plasma membrane, which then raises the hypothesis that unidentified sorting adaptors selectively bring MyD88 to the appropriate membrane region to form signal initiation complexes with activated IL-1 and IL-18 receptors.

In this study, we sought the sorting adaptor for IL-18 signaling and discovered that TRAM is responsible for this function. TRAM was demonstrated to directly interact with MyD88 in *in vitro* binding experiments in which a homotypic TIR-TIR interaction plays a vital role. The efforts to identify the interacting sites in the MyD88-TIR interaction revealed that two surface sites of MyD88-TIR are direct interfaces with TRAM-TIR. Interestingly, these interaction sites overlap with the sites for Mal binding [12]. Furthermore, cellular assays demonstrated the functional involvement of TRAM in the IL-18 signal transduction, and TRAM changed the localization of MyD88 from the cytosol to the membranous regions. These new findings strongly suggest that TRAM is the membrane-sorting adaptor for MyD88 in IL-18 signaling and plays a critical role in transmitting the signal. Thus, the mechanism of signal initiation is more conserved between the MyD88-dependent TLR4 pathway and IL-18 signaling than previously thought.

Results

Binding of TRAM to MyD88

Five TIR containing adaptor proteins have been previously identified: MyD88, Mal, TRIF, TRAM and SARM. Mal and TRAM were reported to be the sorting adaptors for MyD88 and TRIF, respectively [13]. Because a model structure of the TIR domain of TRAM represents a substantially large negatively charged surface area (Figure S1), while the TIR domain of MyD88 is covered by positive charge, we expected some interaction

between these two adaptor molecules. We thus further hypothesized that TRAM also functions as the sorting adaptor for MyD88 in the IL-18 signaling pathway. To test these ideas, we first examined the direct interaction between MyD88 and TRAM. As both of the adaptors contain the TIR domain, which mediates the protein-protein interaction generally via homomeric or heteromeric TIR-TIR interactions, the direct interaction between the MyD88-TIR and the TRAM-TIR was examined with a GST-pull down assay. The results indicated that the wild-type MyD88-TIR directly bound the TRAM-TIR with a higher affinity than Mal, while the interaction between MyD88-TIR and TLR1-TIR, which had been shown not to bind the MyD88-TIR [14], was not detected in this method (Figure 1A). We then examined the interaction between MyD88 and TRAM in cells using a co-immunoprecipitation analysis. When Myc-MyD88 and FLAG-TRAM were co-expressed in HEK293 cells, the Myc-MyD88 constitutively associated with the FLAG-TRAM (Figure 2), which is consistent with our GST-pull down assay. Strikingly, upon stimulation of the cells with IL-18, the MyD88-TRAM complex gradually dissociated over a 30- to 120-minute time course. The HEK293 cells inherently express IL-18R α (formerly called IL-1Rrp), which is a necessary component for IL-18 signaling, but lack IL-18R β (formerly called IL-1AcPL). Thus, we additionally co-expressed IL-18R β in the cells for this experiment.

Binding of TRAM to IL-18 Receptors

For the activation of IL-18 signaling, the heterodimerization of two IL-18 receptors, IL-18R α and IL-18R β , has been shown to be required [15]. These receptors belong to the IL-1 receptor superfamily and are thus structurally homologous to one another. The extracellular region contains immunoglobulin (Ig)-like chains, while the cytosolic region has a TIR domain that interacts with the TIR domain of MyD88. To test the interaction of TRAM with these IL-18 receptors, we performed GST pull-down assays using the TIR domains of the IL-18 receptors and TRAM. The results showed that the TIR domains from both IL-18R α and IL-18R β directly bound the TRAM-TIR (Figure 1B).

Involvement of TRAM in IL-18 Signaling

After obtaining the evidence that TRAM interacts with MyD88 both *in vitro* and in cells, we then examined the possible involvement of TRAM in IL-18 signaling. We knocked-down the endogenous TRAM expression in HEK293 cells using siRNA techniques and then performed NF- κ B reporter assays for the IL-18 signal transduction in the cells. The shRNA for the TRAM expressing vector was generated based on the previously reported target sequence [16]. When the expression of TRAM was knocked-down (Figure 3A), NF- κ B activity after IL-18 stimulation was markedly decreased relative to the negative control experiments that used scrambled shRNA (Figure 3B). This result indicates that the knock-down of TRAM expression actually impaired the IL-18 signal transduction. We confirmed the knock-down effect of this shRNA sequence for TRAM via the LPS-stimulated activation of IFN- β promoter, which is presumably due to the suppression of the MyD88-independent TLR4 pathway mediated by TRAM and TRIF (Figure 3C). The effect was similar when compared to the results obtained from a dominant negative form of TRAM (C117H). TRAM (C117H) showed an almost complete shutdown of the LPS/TLR4/IFN- β signaling in the cells (Figure 3D) and the decrease of the enhancement of NF- κ B activity induced by IL-18 (Figure 3E). To further confirm the involvement of TRAM in the IL-18 signaling pathway, we evaluated the cytokine production from helper type 1 differentiated T (Th1) cells isolated from TRAM-deficient mice and

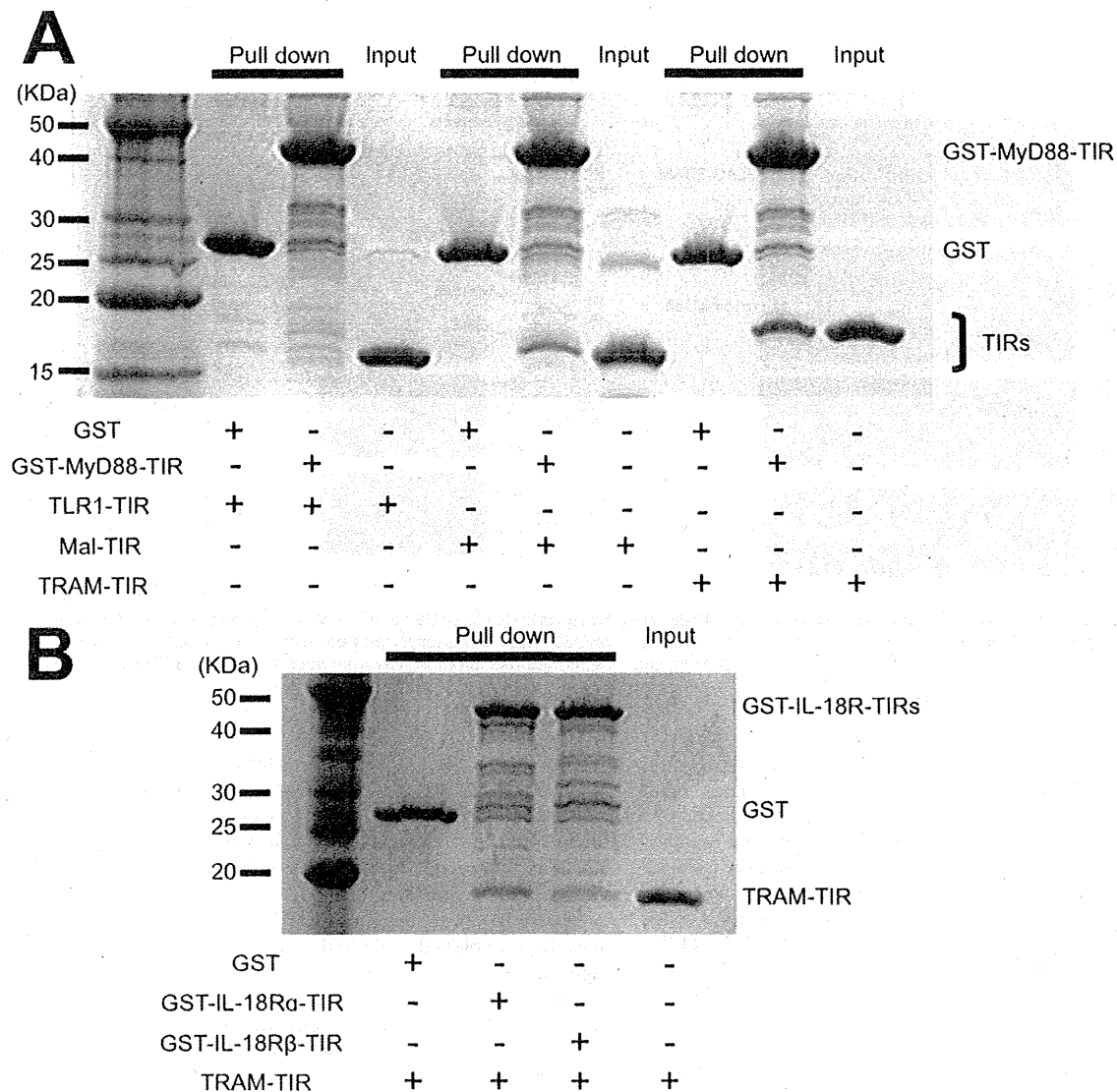


Figure 1. Protein interaction assays. (A) GST pull-down assay investigating the direct interactions between the GST-MyD88-TIR and the TRAM-TIR or TLR1-TIR. (B) GST pull-down assay between the GST-IL-18 receptor TIRs and TRAM-TIR.
doi:10.1371/journal.pone.0038423.g001

MyD88-deficient mice. IL-18 did not produce IFN- γ from not only MyD88-deficient Th1 cells but also TRAM-deficient Th1 cells, significantly. And, IL-18 alone or IL-18 and IL-12 co-stimulated TRAM-deficient Th1 cells produced significantly lower IFN- γ levels than those of wild-type Th1 cells (Figure 4).

IL-18 Modulates MyD88-TRAM Subcomplex in Human Cells

Next, we further investigated whether MyD88 actually interacted with TRAM in human HEK293T cells by live cell fluorimaging. When we transiently transfected a DsRed-TRAM construct into HEK293T cells, the protein localized to the plasma membrane region, which was consistent with a previous report [10] (Figure 5A). In contrast, GFP-MyD88 was dominantly found as foci in the cytosol in the cells expressing the protein; this has also been shown by others [17] (Figure 5B). Strikingly, when

HEK293T cells were co-transfected with expression plasmids for GFP-MyD88 and DsRed-TRAM, MyD88 proteins moderately co-localized with TRAM in the membrane regions (Figure 5C). These data strongly suggest that this transient interaction between the two proteins dramatically altered the localization of GFP-MyD88 from the cytosol to the membranes. Thus, TRAM both bound and endowed MyD88 with membrane targeting properties as has been previously demonstrated for Mal [5].

Identification of Important Amino Acid Residues of the MyD88-TIR in IL-18 Signaling

Having established that MyD88 and TRAM directly interact and that the interaction is critical in IL-18 signaling, we next carried out experiments to identify the amino acid residues of MyD88 that are important in this interaction. A cell-based reporter assay system was utilized to examine various mutations in

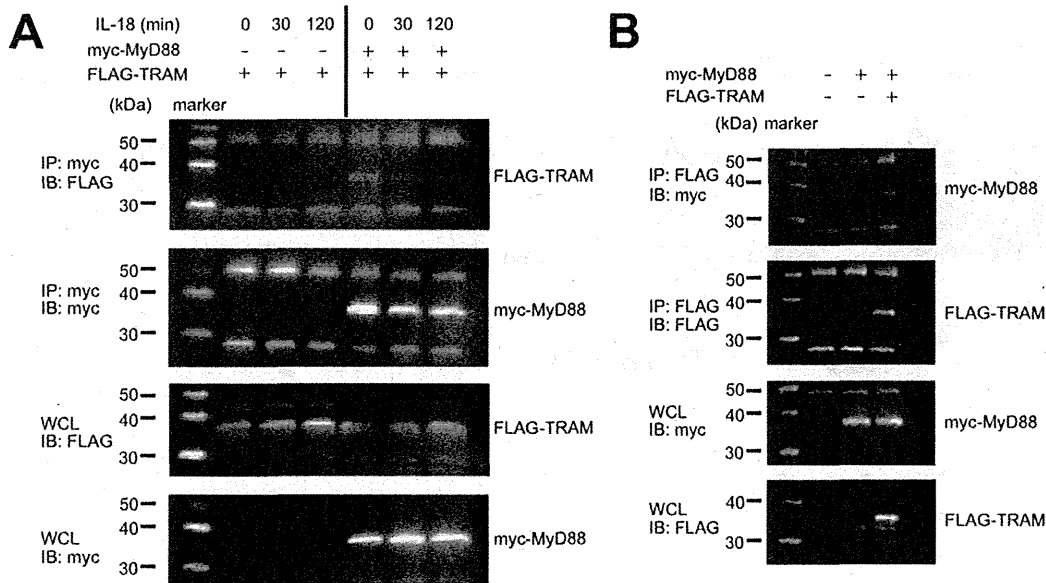


Figure 2. Assay of the interaction between MyD88 and TRAM in IL-18 signaling. (A) MyD88 and TRAM were co-expressed in HEK293T cells along with IL-18R β . IL-18 stimulation was carried out as indicated. MyD88 was immunoprecipitated using the Myc-tag antibody; co-immunoprecipitated TRAM was also detected. (B) The opposite direction co-immunoprecipitation assay between MyD88 and TRAM. Myc-MyD88 was detected with immunoprecipitated FLAG-TRAM. doi:10.1371/journal.pone.0038423.g002

the MyD88-TIR as previously reported [12]. The results are shown in Figure 6A. An alanine substitution of any one of eight residues (Arg196, Asp197, Lys214, Arg217, Lys238, Arg269, Lys282 or Arg288) caused significantly reduced dominant negative inhibitory effects on IL-18 signaling indicating that these residues are involved in the signal transduction. These eight residues are mapped on the protein structure of the MyD88-TIR (PDB code: 2z5v) (Figure 6B). In our previous experiments for LPS/TLR4 signaling using the same luciferase reporter system, three discrete functional sites were found on the surface of the MyD88-TIR, which we designated Site I, Site II, and Site III (Figure 6B) [12]. Five out of the eight residues, Arg196 (Site II), Asp197 (Site II), Arg217 (Site I), Lys282 (Site III) or Arg288 (Site III) were previously found to be important in the LPS/TLR4 signaling. We then examined the direct binding of the representative mutants of each functional sites of MyD88-TIR to the TRAM-TIR using GST pull-down assays (Figure 6C). The results indicate that the binding between the MyD88-TIR and TRAM-TIR is dependent on Sites II and III because the alanine substitution of either Arg196 or Arg288 resulted in decreased binding. Furthermore, the interaction between the MyD88-TIR and TRAM-TIR was completely abrogated when both Arg196 and Arg288 were mutated. Additionally, co-immunoprecipitation assay also showed the reduction of interaction between MyD88 R196A-R288A mutant and TRAM (Figure 6D). In contrast, a Site I mutant, R217A, did not show a significant decrease in binding affinity. Overall, these interactions are very similar to those observed between the MyD88-TIR and Mal-TIR [12], indicating that TRAM and Mal share the same binding sites on MyD88-TIR.

Discussion

Involvement of TRAM in the IL-18 Pathway

Mal has been reported to function as the sorting adaptor in the TLR4 signaling that brings MyD88 to the plasma membrane to

mediate the interactions between TLR4 and MyD88 [5]. Because the IL-1 and IL-18 receptors also form signal initiation complexes that contain MyD88, presumably at distinct membrane regions from TLR4, the existence of currently unidentified sorting adaptors that recruit MyD88 to specific membrane regions and mediate the interactions between MyD88 and the IL-1/IL-18 receptors has been hypothesized [5]. Although several reports have been published to date, the involvement of TRAM in IL-1 signaling remains controversial [16,18,19,20]. Using TRAM-deficient mice, Yamamoto *et al.* showed that TRAM is not involved in IL-1 signaling. Despite the homology between IL-1 and IL-18, the relationship between TRAM and IL-18 had not yet been elucidated. In this study, we sought the sorting adaptor that acts in IL-18 signaling and found that TRAM fulfils this role. It was proposed that the electric potential is important for the specific interactions between the TIR domain proteins [21]. According to the electric surface potentials of the TIR domain structures, the TRAM-TIR and Mal-TIR both have a largely acidic surface patch, while the MyD88-TIR has a largely basic surface patch (Figure S1). Therefore we hypothesized that TRAM interacts with MyD88 and works as the sorting adaptor that recruits MyD88 in IL-18 signaling. In fact, TRAM has already been reported as the sorting adaptor in the MyD88-independent TLR4 pathway in which TRAM recruits TRIF to specific membrane regions [10,22]. Nonetheless, in this work we obtained multiple results that indicate that TRAM functions as the sorting adaptor for IL-18 signals as we initially hypothesized. First, TRAM bound to MyD88 *in vitro* and in cells (Figure 1A, 2, and 5C). Second, the intracellular TIR domains of IL-18 receptors also bound to TRAM-TIR (Figure 1B). Finally, the shRNA knock-down of TRAM expression and knock-out of TRAM caused a significant decrease in the cellular response to IL-18 stimulation (Figure 3 and 4). These findings strongly suggest that TRAM functions as the sorting adaptor for MyD88 in IL-18 signaling. On

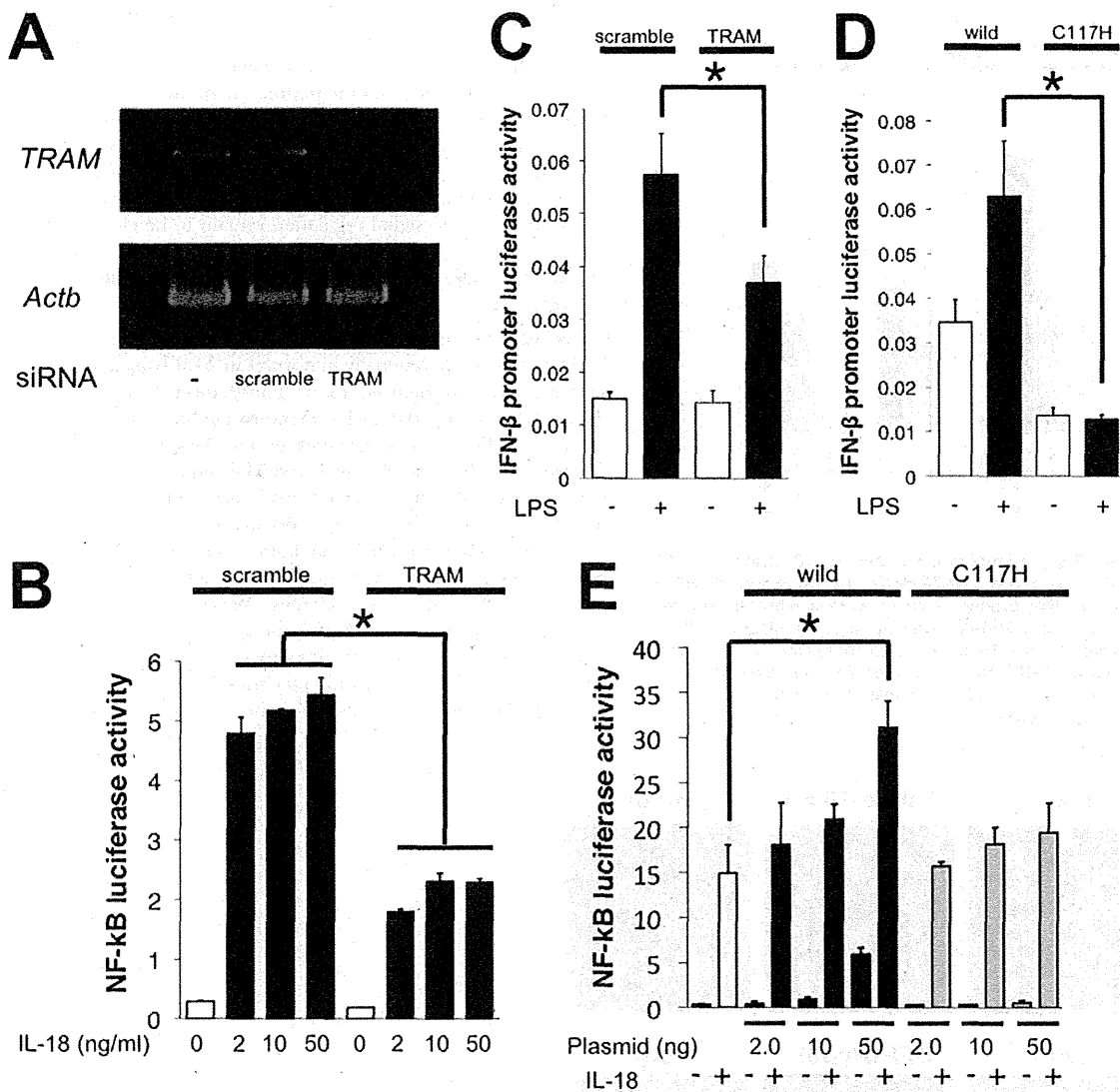


Figure 3. The knock-down effects of TRAM in IL-18 or LPS/TLR4 signaling. (A) RT-PCR of the *TRAM* mRNA knock-down in HEK293T cells by the siRNA-TICAM-2 but not by the nonspecific scrambled sequence coded siRNA vector. "-" indicates cells not transfected with siRNA; "*Actb*" indicates the loading control (β -actin mRNA). (B-E) The effect of the knock-down of TRAM using shRNA or the dominant negative form of TRAM (C117H) for the IL-18 or LPS induced NF- κ B or IFN- β -promoter luciferase activity assay. The NF- κ B and IFN- β -promoter activities with IL-18 or LPS stimulation were significantly decreased in the IL-18 β co-transfected HEK293T cells or HEK293-hTLR4-MD2-CD14 cells. doi:10.1371/journal.pone.0038423.g003

the other hand, recent reports have shown that TRAM is associated not only with TLR4 but also with the TLR2 and TLR5 signaling pathways [23,24]. These findings suggest that the conventionally accepted definition of the functions of the TIR domain-containing adaptor proteins in the TLR and IL-1/18 signaling pathways should be reconsidered.

Time Dependent Change of the Interaction between MyD88 and TRAM

A particularly interesting feature we observed in this study was that the complex between Myc-MyD88 and FLAG-TRAM expressed in HEK293T cells decreased after IL-18 stimulation in a time-dependent manner (Figure 2). This observation suggests that either the rearrangement of the signal initiation complexes or

the degradation of the components in the complexes is triggered by the activation of the IL-18 signaling. A similar rearrangement of the signal initiation complex has also been observed for the TLR4 pathway in a previous report; upon activation of TLR4 by LPS, TLR4 associated with MyD88 instantly, and the association was lost within 15 min [25]. Thus, such transient interactions between the receptors and adaptors and the subsequent loss of the interaction may be common to both the TLR and the IL-18 pathways especially because they utilize many of the same intracellular components. For the TLR4 complex, phosphorylation of TLR4 and Mal has been suggested to be involved in the rearrangements [25,26]. TRAM has been shown to be phosphorylated by Protein kinase C- ϵ (PKC- ϵ) upon stimulation by LPS, and the phosphorylation has been implicated in regulating the

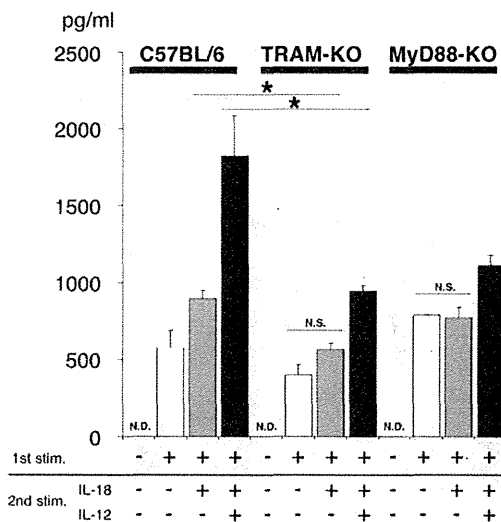


Figure 4. The IFN- γ production from IL-18 and/or IL-12 stimulated Th1 cells from TRAM-deficient mice and MyD88 deficient mice. The IFN- γ production levels were significantly reduced in TRAM deficient mice and MyD88 deficient mice. The black bars show the production levels from IL-18 and IL-12 co-stimulated Th1 cells, grey bars show those from IL-18 solely stimulated Th1 cells, and the white bars show those from no secondary stimulated Th1 cells. doi:10.1371/journal.pone.0038423.g004

myristoylation state and thus the membrane targeting [10,27]. The membrane targeting of another myristoylated protein, MARCKS, has been shown to be regulated by phosphorylation; MARCKS is released from the plasma membrane upon the PKC mediated phosphorylation of a serine near its myristoylation site [28]. Similar to these examples, TRAM might be phosphorylated in the IL-18-induced dissociation of the MyD88-TRAM complex (Figure 2), although the mechanism underlying the dissociation and its relevance to signal regulation remain to be elucidated.

The TRAM Interaction Sites of MyD88 are Similar to that for Mal

According to our previous study [12], Sites I, II, and III on the MyD88-TIR are functionally important in TLR4 signaling. These sites also were recognized to be important in IL-18 signaling (Figure 6A and 6B). We further demonstrated that Sites II and III act as the TRAM binding sites of the MyD88 TIR domain (Figure 6C), which overlap with the Mal binding sites [12]. The two sites are distantly located from each other on opposite molecular surfaces of the protein. Because of the molecular size of the TIR domain, it is unlikely that both sites present a simultaneous binding interface for a single TRAM-TIR to form a 1:1 complex. It is more likely that each of these sites constitutes a distinct interface for a different TRAM molecule in different binding modes. Consistent with this hypothesis, a mutation of either Arg196 or Arg288 leads to only moderate losses in the binding to the TRAM-TIR in contrast to the fact that simultaneous

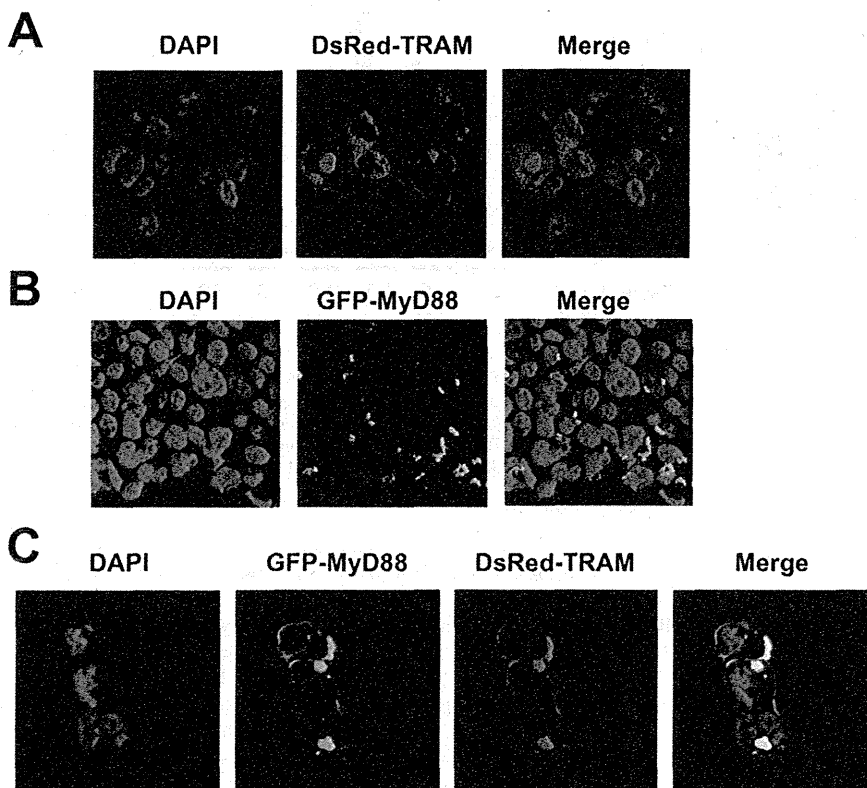


Figure 5. The localization of the MyD88 and TRAM complex in cells. (A–C) The localizations of the DsRed-TRAM (Red) and/or GFP-MyD88 (Green) in HEK293T cells. DAPI stained nuclei of HEK293T cells are shown in blue. Complexes of the DsRed fusion protein and GFP fusion protein are shown in yellow. doi:10.1371/journal.pone.0038423.g005

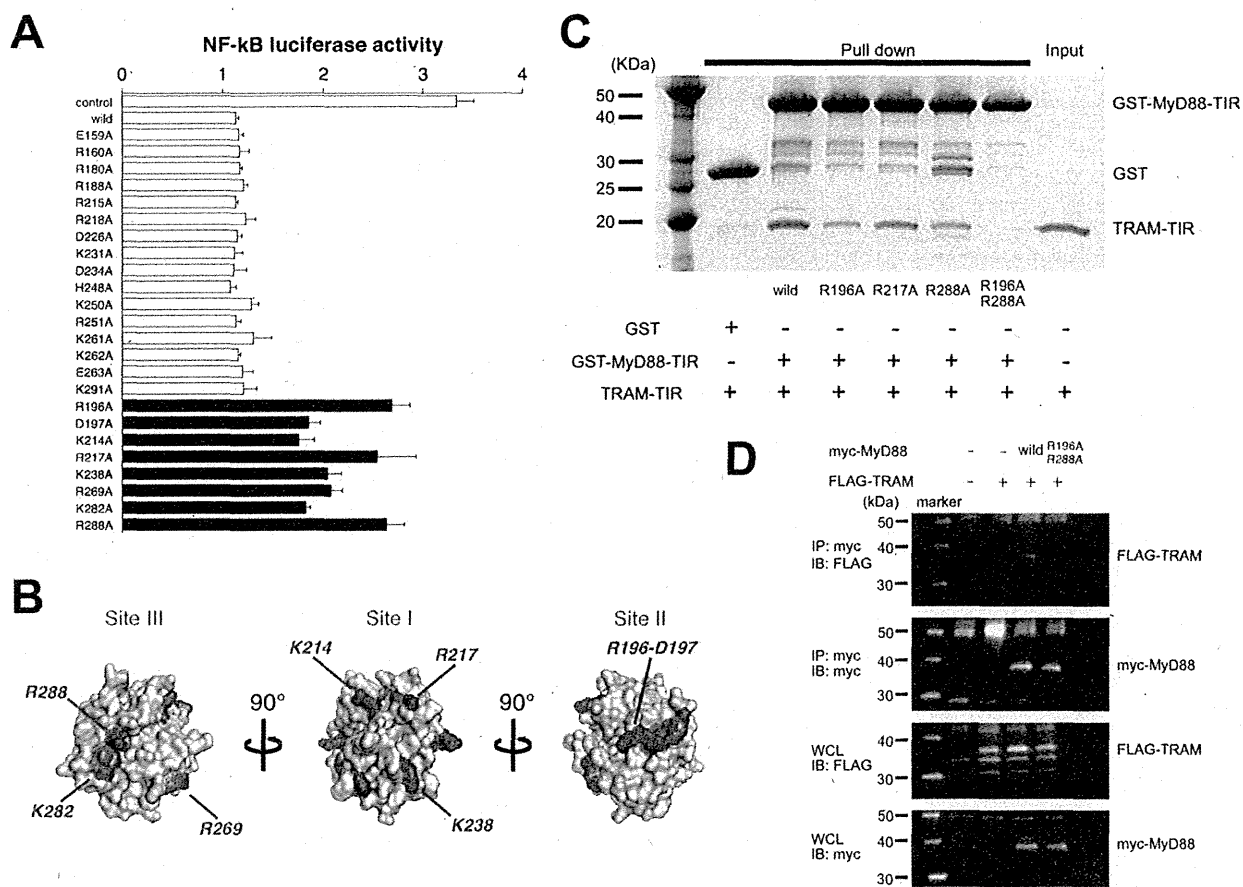


Figure 6. The interaction sites of MyD88 with TRAM. (A) Luciferase reporter gene activities with wild type and mutant types of the MyD88 TIR domain after IL-18 stimulation. The black bars indicate that the residues show significant difference with wild type. (B) The functional assays of IL-18 signaling presented on the 3D structure of the TIR domain of MyD88. Results of the functional assays are mapped onto the molecular surface of the MyD88 TIR domain. The amino acid residues judged to be significant by the luciferase assay are shown in red, while non-significant ones are shown in light brown. The conserved motifs of boxes 1–3 (FDA of box1, VLP of box2, FW of box3) are shown in blue. (C) Assay to study the binding of the wild type or mutant TRAM TIR domain and MyD88 TIR domain. The representative alanine substitutions at Site II (R196A) or Site III (R288A) in MyD88 caused a reduced interaction with TRAM. The double alanine substituted mutant at Site II and Site III caused the complete abrogation of the interaction with TRAM. (D) Immunoprecipitation assay between MyD88 wild or R196A–R288A mutant, and TRAM.
doi:10.1371/journal.pone.0038423.g006

mutations at both residues leads to a total loss of binding (Figure 6C and 6D). With this dual binding mode via Sites II and III, TRAM would be more efficient at recruiting MyD88 to membrane regions. It should be noted that this dual binding mode was also found in the binding between the MyD88-TIR and Mal-TIR [12], which implies that this sort of multiple binding mode is common to the TIR-containing adaptor proteins.

Additionally, in human, the deficiencies of TIR domain containing adaptors, MyD88 and TRIF, have been recently reported [29,30]. These deficiencies were categorized into the innate immune defects. One of the mutations of MyD88, R196C, is known to cause the severe pyogenic bacteria infection due to the loss of interaction between TLR2, Mal and MyD88 [12]. According to the above-mentioned results, Arg196 is one of the binding sites of MyD88 to TRAM. Therefore, the substitution of Arg196 may abrogate not only an initial signaling of TLR mediated by the interaction between Mal and MyD88 but also a secondary enhancement of immune responses mediated by IL-18 induced interaction between TRAM and MyD88 in T cells or NK cells for etiology of human MyD88 deficiency syndrome.

In summary, we have established an unexpected connection between TRAM and IL-18 signaling, which is mediated by a direct TIR-TIR interaction between MyD88 and TRAM, and we proposed that TRAM is the sorting adaptor for IL-18 signaling. Based on the results obtained in this study, we present a schematic model for signal initiation from activated IL-18 (Figure S2) that is similar to the model for the LPS/TLR4 system.

Materials and Methods

Vector Preparations

The following recombinant protein expression cassettes were subcloned into pGEX4T-1, pGEX5X-1 or pGEX5X-3 (GE Healthcare, Buckinghamshire, England): IL-18, IL-1 β , MyD88-TIR (amino acid residues 148–296), TRAM-TIR (66–235), TLR1-TIR (625–786), IL-18R α -TIR (374–541), and IL-18R β -TIR (407–599). A cDNA encoding the MyD88 TIR domain tagged at the N-terminus with a Myc-epitope was cloned into the plasmid vector pcDNA3.1+ (Invitrogen, California, USA). IL-18R β and TRAM constructs tagged at the C-terminus with an

AU1- or FLAG-epitope, respectively, were also cloned into pcDNA3.1+. Mutants of TRAM and the TIR domain of MyD88 were generated using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega, Wisconsin, USA). A pGL3-Basic Vector (Promega) containing four κ B binding sites, which was used in the NF- κ B luciferase reporter assay, and a Renilla luciferase reporter vector used as an internal control in the assay were gifts from Dr. Sewon Ki and Dr. Tetsuro Kokubo (Yokohama City University). An IFN- β promoter region sequence containing pGL4-Luc (Promega) was generated. A pAcGFP-C1-MyD88 (GFP-MyD88) and a pDsRed-Monomer-N1-TRAM (DsRed-TRAM) were also generated (Takara Bio, Shiga, Japan).

Protein Expression and GST Pull Down Assay

The TIR domain of the MyD88 wild type and mutants (R196A, R217A, R288A, and R196A R288A) and the IL-18 receptors (α and β) were purified as GST (glutathione S-transferase) fusion proteins according to methods previously described [1]. The TIR domain of human TRAM and TLR1 was also obtained by a similar procedure as previously described for the MyD88-TIR. These purified proteins were incubated with Glutathione Sepharose 4B (GE Healthcare) for three hours at 4°C, and then these resins were washed four times with wash buffer (20 mM potassium phosphate buffer (pH 6.0), 100 mM KCl, 0.1 mM EDTA, 10 mM DTT, and 0.5% Triton X100), and then analyzed by SDS polyacrylamide gel electrophoresis with Coomassie Brilliant Blue staining. Experiments were performed in triplicate. The mature form of human IL-18 and IL-1 β were prepared using E.Coli expression system according to previously reported methods [15].

Cell Culture

HEK293-hTLR4-MD2-CD14 cells were purchased from Invivogen (California, USA), respectively. HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (high glucose-containing D-MEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (SIGMA-ALDRICH, Missouri, USA), penicillin (100 U/mL) and streptomycin (100 μ g/mL). All cells were incubated at 37°C in a humidified atmosphere of 5% CO₂. The splenic pan T cells were isolated using Pan T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) from the spleen of TRAM-deficient mice, MyD88-deficient mice and background mice (C57BL/6) supplied by Oriental Bio Service (Kyoto, Japan). The purified splenic T cells were incubated with or without 2 ng/ml recombinant murine IL-12 (p70) (PEPRO-TECH, New Jersey, USA) on BIOCOAT anti-mouse CD3 T-cell activation plates (BD Biosciences, Massachusetts, USA) in order to be differentiated into Th1 cells. After 4 days of culture, T cells were washed and restimulated with 20 ng/ml recombinant murine IL-18 (MBL, Nagoya, Japan) and/or 2 ng/ml recombinant murine IL-12 (p70) on anti-mouse CD3 T-cell activation plates for 24 hours. All animal experiments were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals. These cells were cultured in RPMI1640 media (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μ g/mL).

Co-immunoprecipitation Analysis

HEK293T cells in 100 mm plates were transfected with 5.0 μ g of pcDNA3.1+ IL-18R β , 5.0 μ g of pcDNA3.1+ Myc-tagged MyD88 (full-length) wild or R196A R288A mutant and/or 5.0 μ g of pcDNA3.1+ FLAG-tagged TRAM (full-length) using Lipofectamine 2000 (Invitrogen). After 18 hours, the culture media were replaced. After 24 more hours, the cells were incubated with

or without IL-18 (10 ng/mL). These cells were washed with cold PBS and harvested with cell lysis buffer (Tris-HCl buffer (pH 7.5) with 10 mM NaCl, 10 mM EDTA, 0.5% Triton-X100, a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and a phosphatase inhibitor cocktail (PIERCE, Illinois, USA). The soluble cell lysates including 1000 μ g protein were incubated with 5 μ g of anti-Myc antibody (Invitrogen) or anti-FLAG M2 monoclonal antibody (SIGMA-ALDRICH) for 60 minutes; 50 μ l of MultiMACS Protein G MicroBeads (Miltenyi Biotec) that had been equilibrated with cell lysis buffer for 30 minutes at 4°C was then added to the lysates. After incubation, the immune complexes were applied to the magnetic columns. The protein complex samples were then solubilized with 1 \times Laemmli sample buffer after four washes with wash buffer. The samples were analyzed by western blots using an anti-Myc antibody and an anti-FLAG M2 monoclonal antibody.

Knock-down with shRNA or Dominant Negative Mutant of TRAM

The shRNA expression vector psiRNA-h7SKgz-Scr (used as a negative control because it contained a scrambled sequence) and psiRNA-TICAM-2 were purchased from Invivogen. For the reporter gene assays, HEK293T or HEK293-hTLR4-MD2-CD14 cells were seeded at a density of 2.0×10^5 cells/mL per well in a 96-well plate. These cells were transfected with or without pcDNA3.1+ IL-18R β -AU1, NF- κ B luciferase reporter vector, and Renilla luciferase reporter vector with either the psiRNA-h7SKgz-Scr or psiRNA-TICAM-2, pcDNA3.1+ TRAM-FLAG wild or C117H mutant vector using Lipofectamine 2000. After 18 hours, the culture media were replaced with fresh medium, and after an additional 24-hour incubation, the culture media were replaced with fresh medium containing recombinant human IL-18 (2.0, 5.0, 50.0 or 10.0 ng/mL) or LPSO127: B8, which is derived from *E. Coli* strain (100 ng/mL) (SIGMA-ALDRICH), incubated for 6 hours. The luciferase reporter gene activities were analyzed using a Dual-Luciferase Reporter Assay System (Promega). The statistical significance of the differences in the luciferase activities was determined using Dunnett's multiple comparison test. The statistical significance was assigned to be $P < 0.05$.

RT-PCR

Total RNA from cells seeded in six-well plates was isolated with ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Reverse transcription was performed with a 1st Strand cDNA Synthesis Kit (Roche Diagnostics) according to the manufacturer's instructions. The cDNA obtained was used in PCR with Taq DNA polymerase (Toyobo, Osaka, Japan) to determine the relative amount of TRAM mRNA.

ELISA

Culture supernatants in test tubes were centrifuged to remove the cells and then stored at -80°C until analysis. The IFN- γ concentrations were measured using a Mouse IFN- γ Quantikine ELISA Kit (R&D Systems, Minnesota, USA). The statistical significance of the differences in the cytokine productions between the wild type cells and the TRAM or MyD88 deficient cells was determined using two-way ANOVA with Bonferroni's multiple comparison test. The statistical significance was assigned to be $P < 0.05$.

Confocal Microscopy

For direct immunofluorescence, HEK293T cells co-transfected GFP-MyD88 and DsRed-TRAM were washed in phosphate-

buffered saline and fixed for 10 min in 4% paraformaldehyde in phosphate-buffered saline. Cells were then permeabilized with 0.2% Triton X-100 in phosphate buffered saline for 10 min at room temperature. Samples were mounted onto coverslips with Pro-Long Gold Antifade reagent (Invitrogen) and were examined on a Zeiss LSM5 EXCITER confocal microscope. All images were acquired using an aplan-Apochrom at 63X with a 1.4-N.A. objective or at 100X with a 1.4-N.A. objective.

The Screening Method of Functional Residues of MyD88-TIR in IL-18 Signaling

HEK293T cells were transfected with pcDNA3.1+ control vector or pcDNA3.1+ myc-MyD88 TIR domain (wild-type or mutants), pcDNA3.1+ IL-18R β -AU1, NF- κ B luciferase reporter vector, and Renilla luciferase reporter vector using Lipofectamine 2000 according to the manufacturer's instructions. These transfectants were stimulated with recombinant human IL-18 (100 ng/mL) for 6 hours. The luciferase reporter gene activities were also analyzed using a Dual-Luciferase Reporter Assay System (Promega). The statistical significance of the differences was determined using Dunnett's multiple comparison test. The statistical significance was assigned to be $P < 0.05$.

Supporting Information

Figure S1 The surface electrostatic potential of the TIR domain structure models from the TIR domain containing adaptor proteins. These structure models were predicted

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from the template structure of the MyD88-TIR (PDB code: 2z5v) using Discovery Studio 2.6 software (Accelrys). TRAM and MAL have a largely acidic surface patch, while MyD88 has a largely basic surface patch.

(TIFF)

Figure S2 A schematic model of the two distinct regulation patterns in LPS induced TLR4 signaling and IL-18 signaling. MyD88 is efficiently delivered to receptor specific membrane regions by the membrane binding activities of the two associated molecules of Mal or TRAM so that it can form signal initiation complexes with activated TLR4 or IL-18 receptors. Upon stimulation, MyD88 starts to transmit signals through interactions with activated TLR4 or IL-18R, which triggers the phosphorylation cascade mediated by IRAKs and TRAF6.

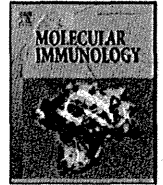
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Acknowledgments

We thank Dr. T. Fukao, Dr. H. Kaneko, Dr. K. Hirayama, and K. Kasahara for their advice and technical help. We thank Dr. S. Ki and Dr. T. Kokubo for their kind gift of vector samples.

Author Contributions

Conceived and designed the experiments: HO HT ZK MS N. Kondo. Performed the experiments: HO HT N. Kawamoto TK KK TY TF HN RW. Analyzed the data: HO HT RW MS. Wrote the paper: HO HT ZK RW MS.



Molecular analysis of the binding mode of Toll/interleukin-1 receptor (TIR) domain proteins during TLR2 signaling

Masatoshi Nada^{a,b}, Hidenori Ohnishi^{a,*}, Hidehito Tochio^c, Zenichiro Kato^a, Takeshi Kimura^{a,d}, Kazuo Kubota^a, Takahiro Yamamoto^a, Yuji O. Kamatari^e, Naotaka Tsutsumi^c, Masahiro Shirakawa^{c,f}, Naomi Kondo^a

^a Department of Pediatrics, Graduate School of Medicine, Gifu University, Yanagido 1-1, Gifu 501-1194, Japan

^b Kumiai Welfare Hospital, Ohshinmachi 5-68, Takayama, Gifu 506-8502, Japan

^c Department of Molecular Engineering, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

^d Neonatal Intensive Care Unit, Department of Pediatrics, Graduate School of Medicine, Gifu University, Yanagido 1-1, Gifu 501-1194, Japan

^e Life Science Research Center, Gifu University, Yanagido 1-1, Gifu 501-1194, Japan

^f Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Hon-cho 4-1-8, Kawaguchi, Saitama 332-0012, Japan

ARTICLE INFO

Article history:

Received 23 September 2011

Received in revised form 23 April 2012

Accepted 1 May 2012

Keywords:

Innate immunity

MyD88

Mal

TIRAP

TIR domain

TLR2

ABSTRACT

Toll-like receptor (TLR) signaling is initiated by the binding of various adaptor proteins through ligand-induced oligomerization of the Toll/interleukin-1 receptor (TIR) domains of the TLRs. TLR2, which recognizes peptidoglycans, lipoproteins or lipopeptides derived from Gram-positive bacteria, is known to use the TIR domain-containing adaptor proteins myeloid differentiating factor 88 (MyD88) and MyD88 adaptor-like (Mal). Molecular analyses of the binding specificity of MyD88, Mal, and TLR2 are important for understanding the initial defenses mounted against Gram-positive bacterial infections such as *Streptococcus pneumoniae*. However, the detailed molecular mechanisms involved in the multiple interactions of these TIR domains remain unclear. Our study demonstrates that the TIR domain proteins MyD88, Mal, TLR1, and TLR2 directly bind to each other *in vitro*. We have also identified two binding interfaces of the MyD88 TIR domain for the TLR2 TIR domain. A residue at these interfaces has recently been found to be mutated in innate immune deficiency patients. These novel insights into the binding mode of TIR proteins will contribute to elucidation of the mechanisms underlying innate immune deficiency diseases, and to future structural studies of hetero-oligomeric TIR–TIR complexes.

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1. Introduction

The innate immune system is the first line of host defense against pathogens. Toll-like receptors (TLRs) are known to be essential sensors of infectious microorganisms by the immune system. TLRs can recognize common molecular structures detected in certain groups of microorganisms, which are described as

Abbreviations: PAMPs, pathogen-associated molecular patterns; MyD88, myeloid differentiating factor 88; Mal, MyD88 adaptor-like; TIR, Toll/interleukin-1 receptor; TRIF, TIR domain-containing adaptor-including IFN- β ; TRAM, TRIF-related adaptor molecule; SARM, sterile α and huntingtin-elongation-A subunit-TOR Armadillo motifs; AP-1, activator protein 1; IRF, interferon regulatory factor; PIP2, phosphatidylinositol 4,5-bisphosphate; IRAK-4, IL-1 receptor-associated kinase-4; NEMO, NF- κ B essential modulator; MyD88-TIR, TIR domain of MyD88; Mal-TIR, TIR domain of Mal; TLR1-TIR, TIR domain of TLR1; TLR2-TIR, TIR domain of TLR2; TLR4-TIR, TIR domain of TLR4; Pam3CSK4, Pam₃Cys-Ser-(Lys)₄; HSQC, hetero-nuclear single quantum coherence; HEK, human embryonic kidney.

* Corresponding author. Tel.: +81 58 230 6386; fax: +81 58 230 6387.

E-mail address: ohnishih@gifu-u.ac.jp (H. Ohnishi).

pathogen-associated molecular patterns (PAMPs) (Akira et al., 2006). PAMPs include bacterial cell wall components such as lipopolysaccharide (LPS), which is contained in the cell walls of Gram-negative bacteria and is recognized by TLR4. Gram-positive bacteria do not carry LPS, but their cell walls contain a thick layer of peptidoglycans and a number of lipoproteins or lipopeptides. These components are recognized by TLR2 and TLR1 or TLR10 (Guan et al., 2010). Intracellularly, TLR signaling is initiated by the binding of various adaptor proteins to the Toll/interleukin-1 receptor (TIR) domains of activated TLRs. Five adaptors containing the TIR domain have been identified: myeloid differentiating factor 88 (MyD88); MyD88 adaptor-like (Mal); TIR domain-containing adaptor-including IFN- β (TRIF); TRIF-related adaptor molecule (TRAM); and sterile α and huntingtin-elongation-A subunit-TOR Armadillo motifs (SARM) (O'Neill and Bowie, 2007). Recruitment of one or more of these adaptors to a TLR initiates signaling events that activate transcription factors such as NF- κ B, activator protein 1 (AP-1), and/or interferon regulatory factor (IRF) families (Kawai and Akira, 2007).

In innate immune responses, MyD88 has pivotal functions in the formation of signal initiation complexes involving the cytosolic domain of TLRs. MyD88 has been reported to be involved in the signaling pathways initiated by all TLRs thus far reported, with the exception of TLR3 (Kaisho and Akira, 2006). Of the MyD88-dependent pathways involving TLR2, -4, -5, -7, and -9, only the TLR2 (including TLR1, TLR6 and TLR10) and TLR4 pathways require Mal for efficient signal transduction (Horng et al., 2002). Recently, Kagan and Medzhitov (2006) revealed that MyD88 and Mal have distinctly different roles in TLR4 signaling: MyD88 serves as an essential “signaling adaptor”, which transmits signals from ligand-activated TLRs to downstream factors to initiate kinase-dependent signaling cascades, while Mal functions as a “sorting adaptor”, which recruits MyD88 to the plasma membrane via its phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain to promote interaction between MyD88 and activated TLR4 beneath the membrane. Indeed, one of our previous studies also suggested that Mal mediates TIR–TIR interactions between TLR4 and MyD88 (Ohnishi et al., 2009).

Thus, while the clear role divisions between MyD88 and Mal in TLR4 signaling have been demonstrated, only limited information is available about their roles in TLR2 signaling. The MAPPIT (Mammalian Protein–Protein Interaction Trap) system showed that Mal was able to enhance both TLR2 and TLR4 signaling (Ulrichs et al., 2007). This finding suggests that Mal is necessary for TLR2 signaling and for the formation of a TLR2/Mal/MyD88 bridging complex. In contrast, other reports have shown that Mal is dispensable for TLR2 signaling at high concentrations of the TLR1/2 ligand, and is only essential at low ligand concentrations (Kenny et al., 2009; Santos-Sierra et al., 2009). Therefore, it has been suggested that Mal acts to enhance TLR2 signaling, but is not essential for transduction of the signal.

Recently, human primary immunodeficiency diseases in innate immunity such as MyD88 and IL-1 receptor-associated kinase-4 (IRAK-4) deficiencies, and ectodermal dysplasia with immunodeficiency have been reported (Picard et al., 2011). In addition, several polymorphisms in TLRs have been reported as being associated with immune disorders (Arbour et al., 2000; Khor et al., 2007). Patients with MyD88 deficiency and IRAK-4 deficiency have been found to be highly susceptible to Gram-positive bacteria, while showing normal resistance to most other kinds of pathogens, such as viruses, widespread bacteria, parasites, and fungi. These phenotypes suggest that TLR2 signaling, which is initiated by peptidoglycan and lipoprotein in Gram-positive bacteria, is more dependent on MyD88/IRAK-4 signaling than other self-defense systems in early life, possibly owing to redundancy in other innate immune signaling pathways. To understand the mechanisms of MyD88 and IRAK-4 deficiencies, it is necessary to analyze the intricacies of the interactions between MyD88 and other signaling components.

Hetero-oligomeric interactions between TIR domains from receptor and adaptor proteins play pivotal roles in these signal transduction systems, as do the hetero-oligomeric interactions of death domain family proteins, but only limited structural information about such interactions is available. Several isolated protein structures of TIR domains have recently been reported (Nyman et al., 2008; Ohnishi et al., 2009; Valkov et al., 2011; Xu et al., 2000); however, the detailed interaction mode of the TIR–TIR domains remains unclear.

Our present study investigated direct interactions between TIR domains involved in TLR2 signaling. *In vitro* binding experiments using purified proteins demonstrated that the TIR domain of MyD88 (MyD88-TIR) directly bound to the cytosolic TIR domain of TLR1, TLR2, and Mal. In addition, we identified four functional residues of the MyD88-TIR that are important for the Pam₃Cys-Ser-(Lys)₄ (Pam3CSK4)-activated TLR1/2 signaling pathway, as well

as for the LPS-activated TLR4 signaling pathway. Furthermore, we identified two binding interfaces of MyD88-TIR for the TIR domain of TLR2 (TLR2-TIR), both of which are shared with Mal. One of these interfaces includes a residue for which a missense mutation causes pyogenic bacterial infections. These analyses of the detailed molecular interaction mechanisms will contribute to elucidation of the etiology underlying recently identified innate immune deficiency diseases and lead to further structural studies.

2. Materials and methods

2.1. Protein sample preparation

The TIR domains of human TLR1, TLR2, MyD88 and Mal were purified using the following modification to previously reported methods (Ohnishi et al., 2009; Tao and Tong, 2009). The portions of the human TLR1 gene encoding the TIR domain (amino acid residues 625–786), the TLR2 gene encoding the TIR domain (amino acid residues 633–784), the MyD88 gene encoding the TIR domain (amino acid residues 148–296), and the Mal gene encoding the TIR domain (amino acid residues 75–235), were each cloned separately into a pGEX-5X-3 vector (GE Healthcare, Buckinghamshire, England). The constructs were transformed into *Escherichia coli* BL-21 (DE3) (Novagen, Darmstadt, Germany). The TIR domains, expressed as GST fusion proteins, were initially purified by glutathione Sepharose 4B FF (GE Healthcare) affinity chromatography, and the GST-tag was removed by digestion with Factor Xa (GE Healthcare). Subsequently, the TIR domains were purified by gel filtration (Sephacryl S-200 HR 16/60 column; GE Healthcare, Buckinghamshire, England). ¹⁵N-labeled TIR domain proteins were prepared using the same purification protocol. All NMR samples were uniformly ¹⁵N-labeled and prepared in 250 μl solutions of H₂O/D₂O (90%/10%) containing 20 mM potassium phosphate buffer at pH 6.0, 10 mM DTT and 0.1 mM EDTA. The ¹⁵N-labeled protein concentration was approximately 0.02 mM for all of the NMR samples.

2.2. NMR spectroscopy

Two-dimensional (2D) ¹H–¹⁵N correlation spectrum for the TIR domains of TLR1 was measured using a hetero-nuclear single quantum coherence (HSQC) pulse sequence on a Varian Innova 900 MHz spectrometer. The 2D ¹H–¹⁵N correlation spectrum for the TIR domains of TLR2 was measured using a HSQC pulse sequence on a Bruker Avance 800 MHz spectrometer equipped with a cryogenic probe. The 2D ¹H–¹⁵N correlation spectra of the TIR domains of MyD88 (wild-type and mutants) and Mal were measured on a Bruker Avance II 700 MHz spectrometer equipped with a cryogenic probe using a HSQC and a SOFAST-HMQC pulse sequence (Schanda and Brutscher, 2005), respectively. All spectra were recorded at 25 °C. For the HSQC spectrum of the TIR domain of TLR1, the matrix size acquired was 100 (t1) and 1864 (t2) complex points with spectral widths of 2553.72 Hz (F1) and 14,383.32 Hz (F2). For the HSQC spectrum of the TIR domain of TLR2, the matrix size acquired was 128 (t1) and 1024 (t2) complex points with spectral widths of 2432.73 Hz (F1) and 12,019.23 Hz (F2). For the HSQC spectra of the wild-type and mutant TIR domains of MyD88, the matrix size acquired was 64 (t1) and 1024 (t2) complex points with spectral widths of 2128.46 Hz (F1) and 11,261.26 Hz (F2). For the SOFAST-HMQC spectrum of the TIR domain of Mal, the matrix size acquired was 64 (t1) and 256 (t2) complex points with spectral widths of 2483.54 Hz (F1) and 5597.01 Hz (F2). The acquired FID data were apodized with a squared cosine-bell window function and zero-filled to double the data points in both dimensions prior to Fourier transformation. All NMR spectra were processed and analyzed with

NMRPipe (Delaglio et al., 1995) and Sparky (Goddard and Kneller, 1999) software.

2.3. Cell culture

Human embryonic kidney (HEK) 293-hTLR1/2 cells were purchased from InvivoGen (San Diego, CA). Cells were cultured in Dulbecco's modified Eagle's medium (high glucose-containing; InvivoGen) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂.

2.4. Vector preparations

A cDNA encoding the TIR domain of MyD88 (amino acid residues 148–296) or the full-length MyD88 (amino acid residues 1–296), tagged at the N terminus with a myc-epitope, was cloned into the plasmid vector pcDNA3.1+ (Invitrogen, Carlsbad, CA). Similarly, a cDNA encoding Mal (amino acid residues 1–235) was cloned into the plasmid vector pFLAG-CMV6a (Sigma). MyD88-TIR mutants of 37 charged polar amino acid residues (Asp, Glu, Arg, Lys, and His) and full-length MyD88 mutants of Arg-196, Arg-217, Lys-282, and Arg-288 substituted by alanine or cysteine were generated using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega, Madison, WI). The vector pGL4.32[luc2P/NF-κB-RE/Hygro], which was used as an NF-κB luciferase reporter vector, and the vector pGL4.70[hRluc], which was used as a Renilla luciferase reporter vector for an internal control, were purchased from Promega.

2.5. Western blot analysis

Cells were harvested, washed with PBS, then lysed. The lysates were cleared by centrifugation at 15,000 × *g* for 5 min at 4 °C. All extracts were adjusted to contain equal amounts of total cellular proteins, as determined using the Lowry method. The supernatants were separated by electrophoresis on SDS polyacrylamide gels and transferred to nitrocellulose membranes using an iBlot Gel Transfer Device (Invitrogen, Carlsbad, CA). Membranes were blocked for 1 h in 5% BSA in TBST (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl and 0.1% Tween 20), then incubated at room temperature for 2 h with an anti-myc antibody (Invitrogen), anti-FLAG antibody (Sigma), or anti-TLR2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with anti-mouse or anti-rabbit IgG HRP conjugate (Promega) at room temperature for 30 min. Detection was performed using ECL-plus (Amersham, Piscataway, NJ) and lightcapture system AE6970CP (AITO, Tokyo, Japan).

2.6. Expression of MyD88 TIR domain mutants

HEK293 cells cultured in six-well plates were transfected with 1.0 µg pcDNA3.1+ empty vector (mock) or 1.0 µg pcDNA3.1+ myc-tagged MyD88-TIR (wild type or mutants), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 and 48 h, culture media were replaced with fresh media. The cells were then harvested using CytoBuster Protein Extraction Reagent (Novagen). The cell extracts (containing equal quantities of protein) were checked by western blot analysis using an anti-myc antibody (Invitrogen). Mutants with poor expression profiles were not included in subsequent studies to avoid potential misinterpretation of the results (e.g. loss of dominant negative inhibitory effect).

2.7. NF-κB reporter gene activity

293-hTLR1/2 cells were transfected with the pcDNA3.1+ empty vector (mock) or MyD88-TIR (wild-type or mutants), NF-κB luciferase reporter vector, and Renilla luciferase reporter vector, using Lipofectamine 2000. After the transfection, the cells were stimulated with synthetic tripalmitoyl lipopeptide Pam3CSK4 (0.1 µg/ml, InvivoGen), then incubated for 6 h. HEK293T cells were also transfected with the mock or full-length MyD88 (wild-type or mutants) and luciferase reporter vectors using Lipofectamine 2000, and incubated for 24 h. Luciferase reporter gene activity was analyzed using a Dual-Luciferase Reporter Assay System (Promega). The inhibitory effect of each TIR expression mutant was assessed by at least three independent experiments. The statistical significance of the differences observed in the luciferase activities between the wild-type and the mutants in the NF-κB reporter assays was determined using Dunnett's multiple comparison test. Statistical significance was assumed at *P*-values < 0.05.

2.8. GST pull-down assays

The TIR domains of the MyD88 wild-type and its mutants (R196C, R217A, K282A-R288A) were purified as GST-fusion proteins. These expression vectors were generated by sub-cloning the pcDNA3.1+ myc-tagged MyD88-TIR into pGEX-5X-1 (GE Healthcare, Buckinghamshire, England). GST fusion proteins were purified by glutathione Sepharose 4B FF affinity chromatography, and subsequently purified with dialysis membrane to exclude less than 30 kDa. GST-fusion proteins of the MyD88-TIR, Mal-TIR or TLR2-TIR, and purified proteins of the TIR domain of TLR1, TLR2, or Mal were incubated with Glutathione Sepharose 4B (GE Healthcare) in binding buffers (20 mM potassium phosphate buffer (pH 6.0), 0.1 mM EDTA, 10 mM DTT, 0.2% BSA and 0.5% NP-40, or 20 mM potassium phosphate buffer (pH 6.0), 10 mM DTT, and 0.5% NP-40) overnight. After four wash steps using 20 mM potassium phosphate buffer (pH 6.0), 100 mM KCl, 0.1 mM EDTA, 10 mM DTT, and 0.5% NP-40, the resin was analyzed by SDS polyacrylamide gel electrophoresis and Coomassie Brilliant Blue staining.

2.9. Immunoprecipitation assays

293-hTLR1/2 cells were co-transfected with the pcDNA3.1+ myc-tagged MyD88 and pFLAG-CMV6a Mal using Lipofectamine 2000. After transfection, the cells were stimulated with Pam3CSK4 (0.1 µg/ml, InvivoGen). The cells were then lysed using 10 mM Tris-HCl, pH 7.5, 20 mM NaCl, 10 mM EDTA and 0.5% Triton-X100, in the presence of a protease inhibitor mix (Roche Applied Science, Indianapolis, IN). The cell lysates were prepared as indicated above (2.5 Western blot analysis). The solutions containing equal quantities of protein were pretreated with the anti-TLR2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h, and then treated with µMACS ProteinG MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 min at 4 °C. Subsequently, the protein concentrations were loaded onto µ Columns (Miltenyi Biotec). After washing with the lysis buffer containing protease inhibitors, the proteins were eluted with SDS-loading buffer and analyzed by western blot analysis.

3. Results

3.1. NMR analyses of TIR domains and GST pull-downs

Recombinant proteins representing the TIR domain of TLR1, TLR2, Mal, and MyD88 were expressed using an *E. coli* expression system. The 2D ¹H–¹⁵N correlation spectra of these TIR domains

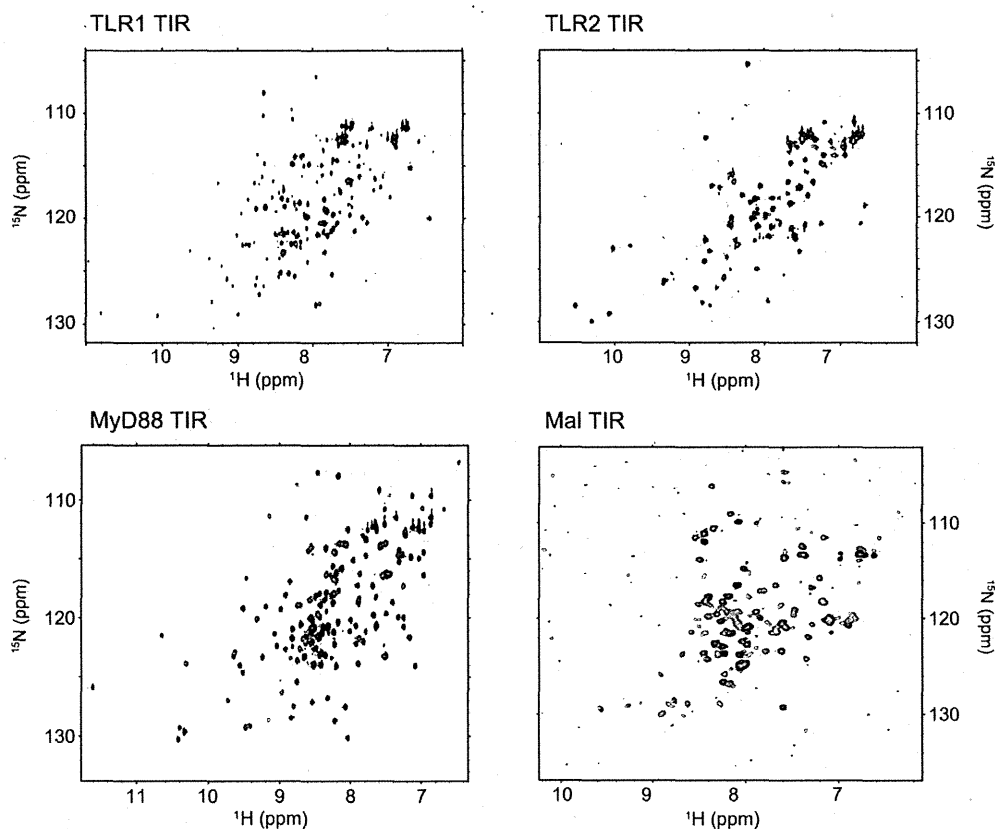


Fig. 1. ^1H - ^{15}N correlation NMR spectra of TLR1-TIR, TLR2-TIR, MyD88-TIR and Mal-TIR. The spectra show the widespread patterns of the backbone amide ^1H - ^{15}N cross-peaks, suggesting that the protein samples had adopted the correct folding of the TIR domains.

showed widespread patterns of the backbone amide ^1H - ^{15}N cross-peaks (Fig. 1), suggesting that the protein samples had adopted the correct folding pattern of the TIR domain. Using GST pull-down assays, we next evaluated direct interactions between the TIR-domain proteins involved in TLR2 signaling. For these experiments, we constructed GST TIR fusion proteins for MyD88, Mal and TLR2. These proteins included no degradation and contaminant bands near the masses of the TIR domains (see Fig. S1). Our previous study showed that the TIR domain of Mal (Mal-TIR) directly binds to the TLR4-TIR, while an interaction between the TLR4-TIR and MyD88-TIR was not detected (Ohnishi et al., 2009). In the present study, TLR1-TIR, TLR2-TIR and Mal-TIR were pulled down with the GST-MyD88-TIR (Fig. 2A). Moreover, the TLR1-TIR and Mal-TIR were pulled down with GST-TLR2-TIR (Fig. 2B), while TLR1-TIR and TLR2-TIR were pulled down with GST-Mal-TIR (Fig. 2C). Hence, these results show that the TIR domain of MyD88, Mal, TLR1, and TLR2 are able to directly bind to each other.

3.2. Functional residues of the MyD88 TIR domain for TLR1/TLR2 signaling

To assess which residues are important for the function of MyD88-TIR, we performed mutational analysis of the domain, using the dominant-negative effects of ectopically expressed isolated TIR domains as a previously reported method for TLR4 signaling (Ohnishi et al., 2009). The expression levels of the MyD88-TIR alanine substitution mutants in HEK293 cells are shown in Fig. 3A. We created 37 alanine substitutions with charged amino acids of the MyD88-TIR. The expression levels of D162A, D171A, E177A, E183A, K190A, D195A, E210A, E213A, D225A, K256A, K258A and D275A were significantly lower than those of the other mutants.

Analysis of the NMR solution structure of the MyD88-TIR showed that the average ratio of the molecular surface exposure of the residues ($20.6 \pm 12.6\%$), of which alanine substitutions were insufficiently expressed, was also significantly lower than that of the other residues ($36.8 \pm 13.5\%$). The differences in the ratio of molecular surface exposure were tested with an unpaired *t*-test; statistical significance was assumed to be $P < 0.05$. The results of the Pam3CSK4 stimulated NF- κ B luciferase reporter gene activity assay, using 25 dominant-negative MyD88 mutants in which expression was confirmed are shown in Fig. 3B. Alanine substitutions of four residues, i.e. Arg-196, Arg-217, Lys-282, and Arg-288, resulted in a significantly reduced inhibitory effect in the Pam3CSK4-induced luciferase activity. Interestingly, these four residues are similar to those involved in TLR4 signaling and closely associated with the box 1, 2, and 3 motifs, which are highly conserved across TIR domains. Arg-196 is located within the box 2 motif. The side chains of Lys-282 and Arg-288 form a continuous protein surface with box 3 forming residues, and Arg-217 is located distant in the sequence, but proximal in space to the box 1 motif. Hence, we recently designated the sites that these residues form as Site II (Arg-196), Site III (Lys-282 and Arg-288), and Site I (Arg-217) (Ohnishi et al., 2009).

3.3. Two interfaces of the MyD88 TIR domain interact with the TLR2 TIR domain

Because Site I, Site II, and Site III of MyD88-TIR are important for TLR2-mediated cellular responses following Pam3CSK4 stimulation, we next examined the involvement of these sites in direct binding to TLR2-TIR. We expressed GST fusion proteins of the MyD88-TIR mutants (R196C, R217A, and K282A/R288A) using