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MyD88 Palmitoylation Mediates Inflammatory Responses in Macrophages

MyD88 팔미토일화 반응에 따른 대식세포 염증반응에 관한 연구

2015년 8월

서울대학교 대학원

분자의학 및 바이오제약전공

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Abstract

MyD88 Palmitoylation Mediates Inflammtory Responses in Macrophages

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Recently, increasingly many proteins are known to be palmitoylated. However, the event of palmitoylatioMyn and its function in TLR signaling have not been previously studied. Here, we demonstrate that myeloid differentiation primary response protein (MyD88) undergoes palmitoylation, a reversible post-translational modification that can regulate protein structure, function, and localization. There are 9 cysteine residues in MyD88 and all of them are highly conserved in closely related species, suggesting the importance of cysteine residue-specific post-translational modifications. MyD88 palmitoylation was confirmed by multiple techniques, including acyl-biotin exchange,

click chemistry, cysteine mutagenesis, and mass spectrometry. Mass spectrometry and cysteine mutagenesis demonstrated that cysteine residue 113 and 274 (C113, 274) were primarily palmitoylated. Palmitoylation of MyD88 was remarkably decreased in mutants where all 9 cysteines were mutated to either alanine or serine (CA or CS, respectively) and also where only C113, 274 were mutated to either alanine or serine (C113, 274A or C113, 274S, respectively). We found that the level of MyD88 palmitoylation correlated with the level of inflammatory responses in HEK293T cells, suggesting the role of palmitoylation in mediating P-p38 and NF-kB signaling. Indeed, both P-p38 and NF-kB activation were significantly inhibited in HEK293T cells stably expressing CA MyD88-GyrB compared to those in WT MyD88-GyrB. We demonstrated that the activation of P-p38 and NF-kB was specific to MyD88-dependent TLR4 signaling by the usage of MyD88-GyrB construct, which only self-dimerizes and being recruited to TLR4 subcellular domain upon coumermycin A1 treatment, excluding the possibility that P-p38 and NF-kB activation is affected by other signaling pathways. We further observed that MyD88 is potentially palmitoylated by DHHC6, a palmitoyl-transferase (PAT) enzyme containing aspartate-histidine-histidine-cysteine (DHHC) motif. A real time quantitative RT-PCR and immunoprecipitation analysis demonstrated that DHHC6 is highly expressed in macrophages and interacts strongly with MyD88 in vitro. Furthermore, acyl-biotin exchange assay showed that shDHHC6-transfected raw264.7 cells failed to palmitoylate endogenous MyD88 in the

absence of DHHC6. These findings suggest that MyD88 palmitoylation is important in mediating inflammatory responses in macrophages.

Keywords: Palmitoylation, inflammation, TLR4 signaling, acyl-biotin exchange assay, macrophages

Student Number: 2013-24025

Contents

Introduction

Lipid modifications, such as prenylation, nitrosylation, and palmitoylation, have been well studied with respect to their roles in protein traficking, stability, and subcellular localization (1). Palmitoylation is the most dynamic one above all; it increasingly has been of interest in the field of proteomics, molecular biology, and biochemistry etc. (1,2). Indeed, a number of popular proteins are known to be palmitoylated such as GPCR (3), cadherins (4, 5, 6), and many other membrane-associated proteins (1).

Palmitoylation is a reversible lipid modification where a 16-carbon fatty acids (palmitate) attaches specifically to a cystein residue of a protein via thioester linkage (S-palmitoylation) (1,2). Although a well-known function of protein palmitoylation is to increase hydrophobicity for membrane association by providing them a suitable configuration for membrane docking, other roles in protein traficking, protein stability and activity have long been remaining elusive due to experimental limitations(7). The study of protein palmitoylation has been accelerated in recent years due to advances in cell-imaging techniques that follow protein trafficking (7). Furthermore, many cytosolic proteins are continuously being identified as palmitoylated, which suggests there is much more to it rather than merely increasing hydrophobicity (2).

Toll-like receptors (TLRs) are key regulators of the immune

response to infectious microorganism (8, 19). Among many, TLR4 is the best-characterized mebmer of the TLR family and is initiated when it recognizes a bacterial cell-surface component lipopolysaccharide (LPS) (11). There are two types of TLR4 signaling pathways named MyD88-dependent and MyD88-independent pathways (12). Both MyD88 and TIRAP are required to activate MyD88-dependent pathway to produce NF-kB and proinflammatory cytokines whereas TRIF and TRAM controls MyD88-independent pathway to induce IRF3-dependent type I interferon production (13, 14, 15, 16, 17).

The relationship between lipid modification, specifically palmitoylation, and TLR signaling has not been previously studied. In this study, we aim to gain insight on the relationship between palmitoylation and inflammation.

Results

MyD88 is palmitoylated

There are 9 cysteines in MyD88 and all 9 cysteines are highly conserved among vertebrates, suggesting the importance of cysteines residue in functional MyD88 (Fig1a). To determine whether TLR4 signaling adaptor molecule MyD88 is palmitoylated, we detected palmitoylated MyD88 proteins using the acyl-biotin exchange (ABE) assay, which exchanges palmitoyl modification with biotin. Hydorxylamine (NH2OH) snaps thioester linkage between the protein and palmitate allowing biotinylation of cysteine residues. Exclusion of hydroxylamine is therefore used as a control for the specificity of biotin labeling. After transiently overexpressing MyD88-GyrB construct in HEK293T cells, we were able to detect MyD88 specifically in the hydroxylamine treated sample suggesting that MyD88 is palmitoylated (Fig 1B). When all nine cysteines are mutated to alanine (CA), a negligible amount of palmitoylated MyD88 was detected in the hydroxylamine treated group (Fig1B). Another method of ABE assay was used to examine MyD88 palmitoylation. IP-ABE assay method is essentially same as the regular ABE method but differs in that MyD88-GyrB is immunoprecipitated first then is subject to acyl-biotin exchange while immunoprecipitated proteins are attached to A/G protein

agarose beads. Again, WT MyD88 in the hydroxylamine treated group showed distinct palmitoylation whereas CA MyD88 showed reduced biotin labeling (Fig1C). In addition, we confirmed MyD88 palmitoylation using a different method named click assay. Click assay uses the commercially available alkyne fatty acid analog 17-octadecynoic acid (17-ODYA) or similarly alkynylated fatty acids, which are metabolically incorporated into endogenous sites of palmitoylation by the cellular palmitoylation machinery (Hang, H.C. et al., 2007). Exclusion of 17-ODYA is therefore used as a control for specific alkynylated fatty acid-labeling. Palmitoylated MyD88 was detected only in the 17-ODYA treated group comfirming MyD88 palmitoylation (Fig1D). Furthermore, we were curious if MyD88 palmitoylation increases upon LPS-treatment. ABE assay demonstrated that the level of MyD88 palmitoylation is not enhanced in response to 40 minute-long LPS treatment (Fig1E).

LC-MS/MS results show MyD88 is palmitoylated at cysteines 113 and 274

The trypsin-digested peptide samples were analyzed using an LC-MS/MS system consisting of a Nano Acquity UPLC system and an LTQ Orbitrap Elite mass spectrometer equipped with a nano-electrospray source (Fig2A-C). Low-confident spectra were obtained using a 7T LTQ FT mass spectrometer (Fig2D-E). Two sequential trials showed that C113 and C274 are

NMM-labeled, respectively (Fig2).

MyD88 is palmitoylated at cysteines 113 and 274

MyD88 has three domains: death domain, intermediate domain, and TIR domain. (Fig3A) An amino-terminal death domain (DD), found in proteins involved in cell death, accounts for MyD88 self association. Intermediate domain (ID) links Death and TIR domain and consists of few amino acids including a palmitoylation site C113. Intermediate domain is known crucial for IRAK4 binding; short MyD88 which lacks ID fails to form a complex with IRAK4 and unable to propagate the downstream signals (24).

We then examined whether mutation in C113 and C274 indeed reduces the amount of palmitoylated MyD88 in vitro. ABE assay was performed using C113, 274S double mutant after transiently over expressing in HEK293T cells. Compared to wild type MyD88, C113, 274S MyD88 showed reduced palmitoylation that is comparable to CS mutant (Fig2B). C113, 274A MyD88 mutant also showed reduced palmitoylated amount, which further confirms two sites of palmitoylation (Fig2C). Hydroxylamine-untreated group was used as a negative control for each sample. Although it seems like non-specific binding of biotin is inevitable to some extent, since hydroxylamine treated samples show significant elevation in amount of palmitoylated

MyD88, the data confirms MyD88 is palmitoylated at C113 and C274.

Palmitoylation of MyD88 mediates P-p38 and NF-kB activation

A proper MyD88 self-association and recruitment of other adaptor molecules to TLR4 subcellular domain initiates MyD88-dependent signaling cascade. Phosphorylation of p38 (P-p38), an active form of p38, is a hallmark of proper MyD88-dependent signaling transduction and we examined the level of P-p38 as to find the functional significance of MyD88 palmitoylation. HEK293T cells stably expressing WT and CA MyD88-GyrB used to prevent base-line activation of MyD88-dependent signaling when MyD88 alone is overexpressed in cells (23). It has been known that MyD88 overexpression in HEK293T cells initiate MyD88 self-dimerization and signal propagation even in the absence of TLR4 recognition of LPS. When GyrB is fused to carboxyl terminus of MyD88 and generate a stable cell line, it serves to prevent MyD88 self-dimerization in HEK293T cells. MyD88-GyrB dimerizes upon binding with the Streptomyces product coumermycin with a stoichiometry of 2:1 and elicits the same response as LPS stimulus. While WT MyD88-GyrB properly stimulates P-38 activation, CA MyD88-GyrB fails to elicit P-p38 response upon

treatment of coumermycin A1, suggesting the importance of palmitoylation upon proper signal transduction (Fig4A). In order to confirm the activation of P-p38 is specific to coumermycin effect, a related monomeric antibiotic, novobiocin, was used. Novobiocin binds GyrB as a 1:1 complex, which inhibits coumermycin binding to MyD88-GyrB. The result shows that P-p38 activation was completely inhibited when cells were pre-treated with novobiocin for 3 hours before treatment of coumermycin (Fig4B), demonstrating that the MyD88-GyrB fusion protein does not exhibit nonspecific activation unless cells are exposed to coumermycin A1 stimulation. We next examined whether NF-kB signaling is affected in CA MyD88-GyrB. After NF-kB plasmid was transiently over-expressed in HEK293T cells stably expressing MyD88-GyrB WT and CA, cells were subject to NF-kB luciferase assay after coumermycin treatment for 20 min. Compared to WT MyD88-GyrB, CA MyD88-GyrB showed significantly reduced NF-kB-luciferase activity, suggesting NF-kB activation is also MyD88 palmitoylation-dependent.

Screening MyD88-specific PAT enzyme

Protein palmitoylation is mediated by a family of palmitoyl-acyl transferase (PAT) enzymes containing a conserved DHHC motif (18). There are 23 mammalian DHHC proteins have been identified (20); only 17 of theses have been shown to have PAT

activity (21). We aim to screen which DHHC protein palmitoylates MyD88. First, we tried to eliminate ones that are not expressed in monocytes. mRNA expression level of each DHHC protein was examined using quantitative RT-PCR in raw 264.7 cells. The results showed almost no expression of DHHC8, 11, 13, 17, 18 and 19 (Fig5A). Thus, we were able to eliminate five DHHC proteins and moved on to immunprecipitation analysis to examine which DHHC enzyme interacts with MyD88 in vitro. We coexpressed each 17 DHHC protein with Flag-MyD88 in HEK293T cells and they were subject to immunoprecipitation with anti-FLAG-agarose beads. The results were repeated at least 2-3 times and a table summarizing the number of binding occurred in the same condition was generated (Fig.5B) The table suggests that DHHC4 and 7 interact with MyD88 to a medium level (66%) while DHHC6 and 22 interact with MyD88 strongly (100%). To confirm whether DHHC 4, 6, 7, and 22 are expressed in human THP-1 cells, quantitative RT-PCR was performed (Fig5C). The data demonstrated that the mRNA expression of DHHC 6 was the highest. Next, we performed immunoprecipitation analysis after Flag-DHHC6 alone is transiently overexpressed in HEK293T cells. Endogenous MyD88 was immunoblotted using anti- human MyD88 antibody. Compared to the negative control, endogenous MyD88 showed interaction with Flag-DHHC6 (Fig5C). Combining information illustrated in Fig. 5A-D, we were able to infer that DHHC6 can potentially be the enzyme that palmitoylates MyD88. To confirm whether DHHC6 indeed participates in MyD88 palmitoylation,

shDHHC6 transfected Raw264.7 stable cell line was used to perform ABE assay. Compared to control Raw264.7 cells, shDHHC6 cells resulted in a reduced amount of palmitoylated MyD88 in vitro (Fig.5E).

Discussion

Our findings imply that MyD88-dependent signaling events are affected by S-palmitoylation, by which innate immune signal transduction might be enhanced in living organisms. However, the effect of short-term LPS treatment did not significantly augment the level of palmitoylation, suggesting that palmitoylation events are far more dynamic than we expect it to be. It could be that the rate of palmitoylation increases upon LPS treatment yet the total amount of palmitoylated MyD88 remains constant through out immune response. We cannot exclude the possibility that the level of MyD88 palmitoylation is rather affected very shortly after stimulation of LPS, which makes us difficult to examine in vitro due to limitation of human error. However, S-palmitoylation of MyD88 is likely to enhance signaling cascades, by which acute-phase signal propagation that leads to inflammatory responses might be affected by rapid turn over of palmitate in MyD88. Such an effect may reflect an adequate regulation of acute-phase inflammatory responses by mediating attachment and detachment of palmitates in vivo.

It has been known that S-nitrosylation, another cysteine residue-specific post-translational modification, influences MyD88-dependent signaling events in an opposite way of our findings suggest (22). The authors of the paper suggest that NO generated from eNOS and iNOS exerts a suppressive effect on

acute-phase inflammatory responses to LPS in vivo probably through S nitrosylation (22). The site of S nitrosylation in MyD88 is suggested to be C216 (22), which does not overlap with our data of C113 and C274 being palmitoylated. However, C113, 216, 274S triple mutant MyD88 showed reduced protein stability upon ABE assay as we were unable to compare the level of palmitoylation due to difference in protein expression level in Input samples. Thus, it remains elusive the precise mechanism that modulates S palmitoylation in relation to S nitrosylation; however, it is clear that S palmitoylation of MyD88 affects the level of inflammatory responses triggered by MyD88-dependent signaling.

We found that P-p38 and NF-kB activation is halted when all 9 Cys residues are mutated to serine or alanaine (Fig 4). However, how MyD88 palmitoylation affects P-p38 and NF-kB activation is remain unstudied. It is possible that palmitoylation of MyD88 influences self-association of MyD88 prior to recruitment in subcellular TIR domain of TLR4. It is also possible that MyD88 palmitoylation is critical in interaction between MyD88 and TLR4 after MyD88 is dimerized. Since C274 is located within TIR domain responsible for interaction with TLR4, we initially thought that MyD88 palmitoylation would primarily responsible for interaction with TLR4. However, mass spectrometry result demonstrated with high confidence that C113 is palmitoylated. C113 is located in the intermediate domain (ID) of MyD88 (Fig 3A) and ID of MyD88 is known critical in regards to interaction with IRAK4 (24) (Fig 6), a downstream molecule necessary for

proper TLR4 signaling transduction (25). Thus, previous findings concerning roles of each MyD88 domain together with our data suggest that MyD88 palmitoyltaion is involved in mediating inflammatory responses in a more complex manner.

Intracellular palmitoylation dynamics are regulated by a family of 23 DHHC (aspartate-histidine-histidien-cysteine) palmitoyltransferases, which are localized in a compartment-specific manner (26). The majority of DHHC proteins localize to endoplasmic reticulum (ER) and Golgi membranes, and a small number target to post-Golgi membranes (26). In this paper, we demonstrated that DHHC6 is probably sufficient to palmitoylate MyD88. DHHC6 contains a predicted Src-homology 3 (SH3) domain and is localized to the ER membrane (27). It has known that DHHC6 forms a complex with selenoprotein K in ER to coordinate ER structure and function (27); beyond that, other roles of DHHC6 has been unstudied previously. Although a large part of DHHC6 involvement in mediating inflammation is not known, our results suggest that DHHC6 participates in MyD88 palmitoylation and imply that DHHC6 may be crucial for mediating inflammatory responses in macrophages. In addition, it is possible that other DHHC proteins such as DHHC4, DHHC7 and DHHC22 may also participate in MyD88 palmitoylation. Further examination is needed to confirm using shDHHC plasmid followed by ABE assay as well as functional assays checking P-p38 and NF-kB activation in the absence of each DHHC enzyme.

Recently, an idea that links cellular lipid metabolism to immune responses has been raised to issue. The question of how accumulated fatty acids can regulate inflammatory responses has recently been answered partly by looking at lipid synthesis by fatty acid synthase (FASN) (28). When FASN is inhibited, IL-18 and pro-IL-1β, pro-inflammatory cytokines, gene expression were suppressed in macrophages (28). Since C-16 palmitate is an initial product of functional FASN, it is highly possible that palmitoylation is affected by the activity of FASN. If it is true, the relationship between FASN and inflammation presented in the previous paper (28) is consistent with our results, where the level of MyD88 palmitoylation results in pro-inflammatory P-p38 and NF-kB activation. Indeed, a different form of fatty acids, polyunsaturated fatty acids (PUFAs), particularly the n-3 series, are used clinically as immunosuppressive agents (29) and in the treatment of various inflammatory diseases (30, 31, 32, 33), suggesting clinical implication using a palmitate analog to lessen an early inflammatory responses.

In conclusion, we here demonstrate that a key adaptor molecule MyD88 in TLR4 signaling is palmitoylated potentially by DHHC6 and as a result downstream molecules such as P-p38 and NF-kB are activated allowing proper inflammatory responses upon LPS challenge. Although there are much more to be explored in terms of precise mechanism of how MyD88 palmitoylation affects inflammation, it is of note that fighting infectious microorganisms in macrophages are indeed dependent on palmitoylation.

Materials and Methods

Cell culture and materials

HEK293T cells were grown in DMEM and Raw264.7 cells were maintained in RPMI medium containing 10% fetal bovine serum with 1% antibiotics at 37℃ in a 5% CO2 incubator. MyD88-GyrB series (WT and various mutants) in pcdna3 plasmid were used and Lipofectamine LTX transfection reagents were used for DNA transfection, following the manufacturer's instruction.

Plasmids

The cDNA of MyD88 was amplified by PCR and cloned into the CAG vector. The DNA construct endcoding MyD88 fused to the B subunit of the bacterial DNA gyrase (MyD88-GyrB) was cloned in pcDNA3.1 vector (Invitrogen) by amplifying MyD88 from the Flag-MyD88. Constructs encoding mutated MyD88-GyrB were obtained using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instruction. The cDNA of MyD88-GyrB was amplified by PCR and cloned into the pcDNA4-V5-A vector (Invitrogen). pEF-bos-HA DHHC plasmids were kind gifts from Fukata M.

(National Institute for Longevity Science, Japan).

Immunoprecipitation

HEK293 cells transiently transfected with recombinant Flag-MyD88 plasmid were washed twice with PBS, and lysed with IP lysis buffer containing 0.1% NP-40, and proteinase inhibitor cocktail (Roche). Cell lysates were incubated on ice for 30minutes and centrifuged 30 min at 4°C. Protein quantification was done by BCA quantification followed by manufacturer's protocol and immunoprecipitated with FLAG-Gel overnight. Beads were washed 4 times by lysis buffer, followed by immunoblot analysis using anti-Flag antibody.

Luciferase reporter assay

HEK293T cells stably expressing wild-type MyD88-GyrB or MyD88-GyrB mutants, each with a cysteine residue replaced with either a serine or alanine residue, were transfected with 100ng of an NF-kB (5x) luciferase reporter plasmid (pNF-kB Luc; Stratagene) and 0.5ng of an internal control luciferase reporter plasmid (pRL-TK; Promega) and incubated for 30 h. At 6 h before the end of incubation, cells were treated with or

without 0.1ug/mL LPS and at 4 h before the end of incubation, cells were treated with or without 0.1ug/mL coumermycin A1.

Acyl-Biotin Exchange (ABE) assay

HEK293T cells were transiently transfected with MyD88-GyrB or MyD88-GyrB mutants and incubated for 48 h. Cells were harvested using palmitoylation lysis buffer containing 1% NP-40, 150mM NaCl, 50mM Tris-HCl pH7.4, 50mM N-ethylmaleimide, PMSF, and proteinase inhibitor cocktail (Roche). Cells were sonicated for 30 sec and rotated at 4°C overnight. Chloroform-methanol (C-M) precipitation was done on cell lysates and concentrated proteins were solubilized in 4% SDS-containing buffer (SB). Solubilized proteins were diluted with hydroxylamine buffer pH7.4 or with palmitoylation lysis buffer pH7.4 as a negative control. Both hydroxylamine buffer palmitoylation buffer contain 1uM biotin-HPDP and proteinase inhibitor cocktail and rotated at RT for 2 h. Lysates were C-M precipitated, solubilized, and diluted again with palmitoylation lysis buffer containing 0.1% triton X-100. They were BCA quantified followed by immuneprecipitation with neutravidin (Pierce). Beads were rotated at RT for 1.5 h and washed 4 times with palmitoylation lysis buffer followed by immunoblot analysis using anti-MyD88 antibody (DF8Q cell signaling).

Click Assay

HEK283T cells were transfected with wild-type MyD88 using Metafectene pro, following the manufacturer's instruction, and incubated for 30 h. Cells were metabolically labeled with 17-ODYA for 10 h. Cells were lysed with lysis buffer containing 1% NP-40 and sonicated for 30 sec. Lysates were C-M precipitated and solubilized in 4% SDS-containing buffer. Tamra-azide 5mM was added for Click chemistry reaction was performed in RT for 1.5 h. Samples were then C-M precipitated again and washed 3 times with methanol followed by BCA quantification. Samples were immunoprecipitated by anti-TAMRA antibody and rotated at 4°C overnight. Protein A/G agarose beads were used to immunprocipitate anti-TAMRA antibody and washed 4 times with washing buffer BC150 and BC300 in the order of BC150, BC300, BC300, and BC150. Beads were eluted with 2X sample buffer containing 4% SDS and DTT followed by immunoblot analysis using anti-MyD88 antibody (Cell Signaling).

Realtime quantitative RT-PCR

RNA was extracted using trizol reagent from Raw264.7 cells. The cdna was generated using AccuScript High Fidelity cDNA synthesis kit. Quantitative RT-PCR was performed using each

DHHC-specific primers listed in table below (Table1). L32 and B2M were used as endogenous controls in Raw 264.7 and THP-1 cells respectively. Bar graph was generated using GraphPad Prism 5 and presented on average of triplicates.

Mass Spectrometry (MS)

HEK293T cultures were lysed at 30 h following transfection with Flag-MyD88, solubilized and Flag immunoprecipitated with anti-flag affinity gel (sigma) in the presence of 50mM NEM. The beads were rotated at 4 overnight and washed three times with IP lysis buffer. Next, the beads were treated with hydroxylamine buffer pH7.4 followed NMM labeling at 4 for 2 h. Beads were washed three times, eluted, and separated by SDS-PAGE. Gel was stained by colloidal blue reagent at RT overnight and destained by destaining buffer (methanol-containing) at RT overnight. MyD88 specific bands were cut and sent to Korea Basic Science Institute (KBSI).

<Table1>

Primer name	5' - sequence -3'
dhhc1 F	TACCGCGAGGGTCTGAGG
dhhc1 R	GCTGACTTGAAAACAGCTGCG
dhhc2 F	TACTGGATCCCGGTGGTGTT
dhhc3 F	TGGTGTACAAGTGCCCCAAA
dhhc3 R	TGTACATTGTAAACAGGACGAAGT
dhhc4 F	CTCCCTTTCCCGTTGTGTGT
dhhc4 R	GGCATTCGTCTTCCCAACAT
dhhc5 F	TTCTTGTGTGTTCTGCCGCT
dhhc5 R	AGTCCTGGACACGTAAAGGC
dhhc6 F	TGTCCATGACGGAGTCATCC
dhhc6 R	TCAGTGTGGTCCCTGGTGTC
dhhc7 F	GATGAATCCCTTTGTGGGCT
dhhc7 R	GAGCGTCTTCCTCTGCCATC
zdhhc8 F	CTTCTTCGTGTTCACGTGCC
zdhhc8 R	TCCATGAAAGTGGCCATGCT
dhhc9 F	CTGCTTCCCGACGGATTTTG
dhhc9 R	CCAATTGCTAGCCCTGGAAGA
zdhhc10 F	TTGTCAGGAGGAGACAGCCT
zdhhc10 R	GAGCCGGCCTGAACCAC
zdhhc11 F	CCAGCTGCTCATCTTCCACA
zdhhc11 R	GCTCTTGGCTTTGACTCCCT
zdhhc12 F	GGAGAACTGTGTGGGAGAGC
zdhhc12 R	GGCCTGACCATGCCAGGTA
dhhc12 F	TCACTCATGGACCCTGGCTA
dhhc12 R	CACAAAGAGTGGGTGGTTGC
zdhhc13 F	CTTGCCTAGTAGCCTCAGCC
zdhhc13 R	TGTGATTCCTGCACTGCGA
zdhhc14 F	CCACAGCTCCTCCCCCAT
zdhhc14 R	GGTACGGACAGTCGAAGGC
zdhhc15 F	TGAGTCACAGAACCCACTGC
zdhhc15 R	TCCCCTAGGAAAATACTGTCTGA
dhhc16 F	TGCCAACCAGACTTATCACCA
dhhc16 R	AACTGCACAGGAACCAGAGG
dhhc17 F	CATTCAATGTTTCAGTTAACCTTGG
dhhc17 R	CGCTGATTCGCCCTTGATATT
dhhc18 F	CCCCTACCTCCCAGCCTAAT
dhhc18 R	CAGCAGGTGGCAAGTACTGA
zdhhc19 F	GCCTCTTCTTCGCATTCCCT
zdhhc19 R	CTCAGCGGAGCCTTGATGTA
zdhhc20 F	CAAGCTTGGGTGATGGTTGC
zdhhc20 R	TGATTTGAGCCACTTCTGGCA
zdhhc21 F	CATGCGGACACCCACTCG
zdhhc21 R	GTTCTTCATTCGTCGCCGC
zdhhc22 F	CGAGTCCACAACTTTGCAGC
zdhhc22 R	TACATTCCGAGCTCTGGTCG
dhhc23 F	AAGGGCAGGAGAAGACCAAA
dhhc23 R	CACTTGGCACACCAGTCCTC
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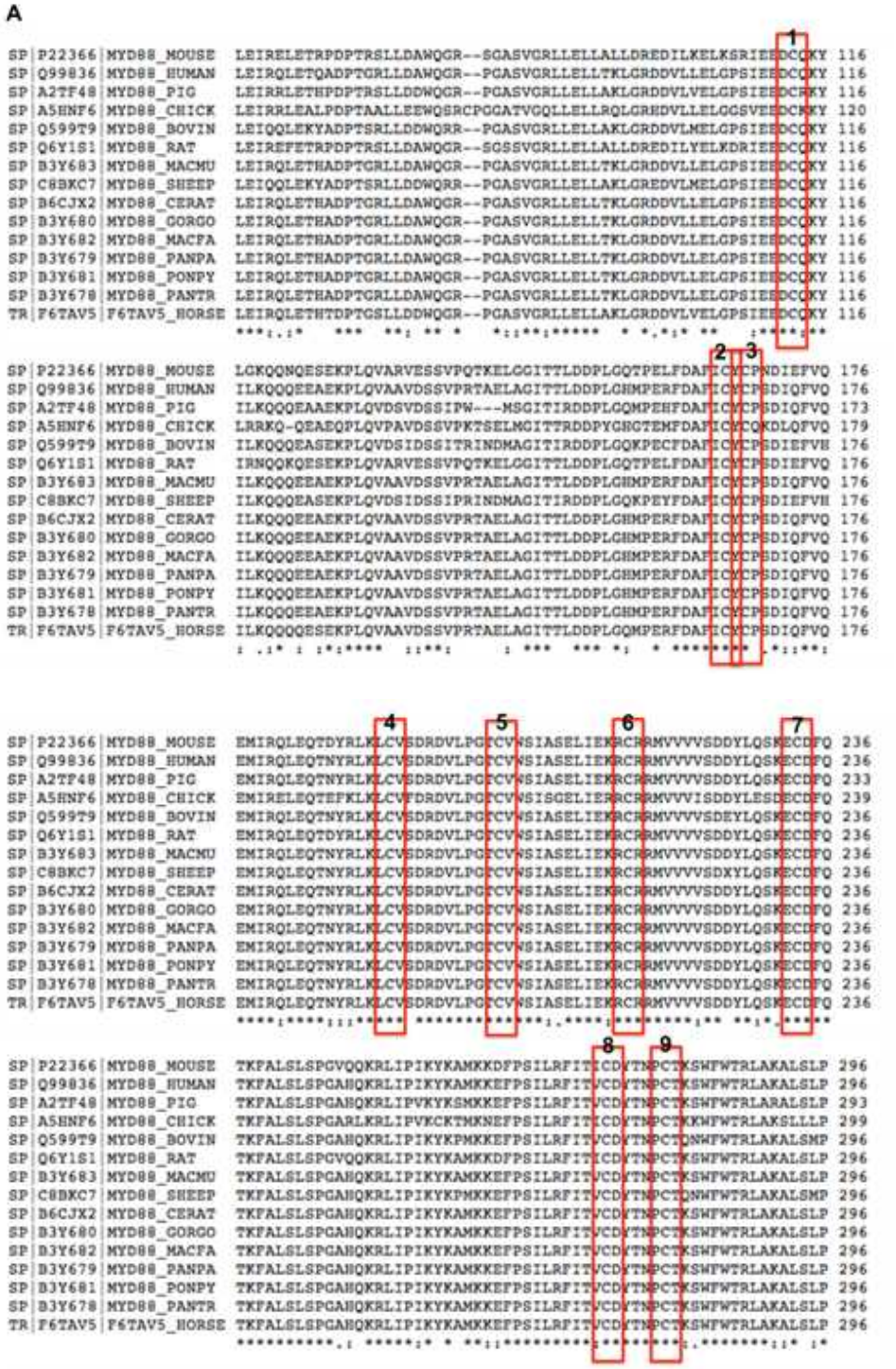
zdhhc24 R	GGGCTTTAAGGCTCTGCAAAT

Figures



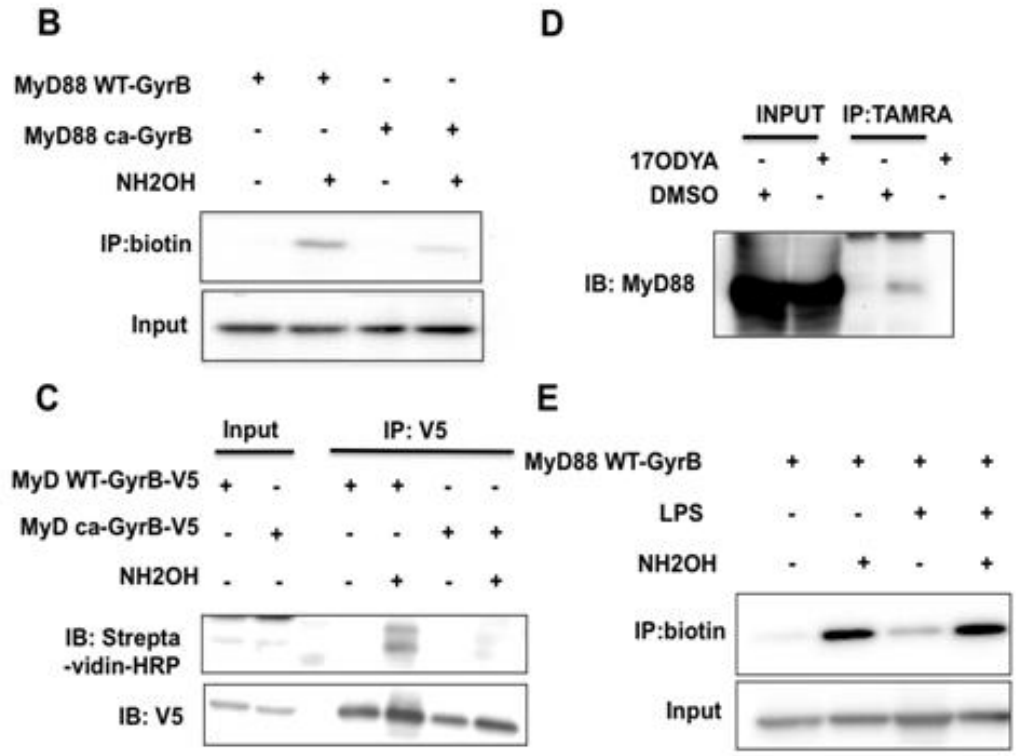


Figure 1. MyD88 is palmitoylated.

A) The protein sequences of MyD88 in various species were aligned using Uniprot alignment application. All 9 Cys were highly conserved among close-related species. Red box indicates conserved cysteine residues and individual cysteine residue is labeled 1 through 9. B) Acyl-biotin exchange assay revealed by western blotting. The amino acids mutated from cysteine to alanine are indicated (CA). HEK293T cells were transfected with wild-type and mutant MyD88-GyrB (n=10). C) HEK293T cells stably expressing wild-type and mutant MyD88-GyrB were subject to IP-ABE assay and revealed by western blotting (n=2). D) HEK293T cells were transiently transfected with wild-type MyD88 and were subject to Click assay. D) Acyl-biotin exchange

assay revealed by western blotting. 0.1ug/mL LPS was treated for 40 min. after 30 h of transient transfection of MyD8-GyrB incubation. (n=2)

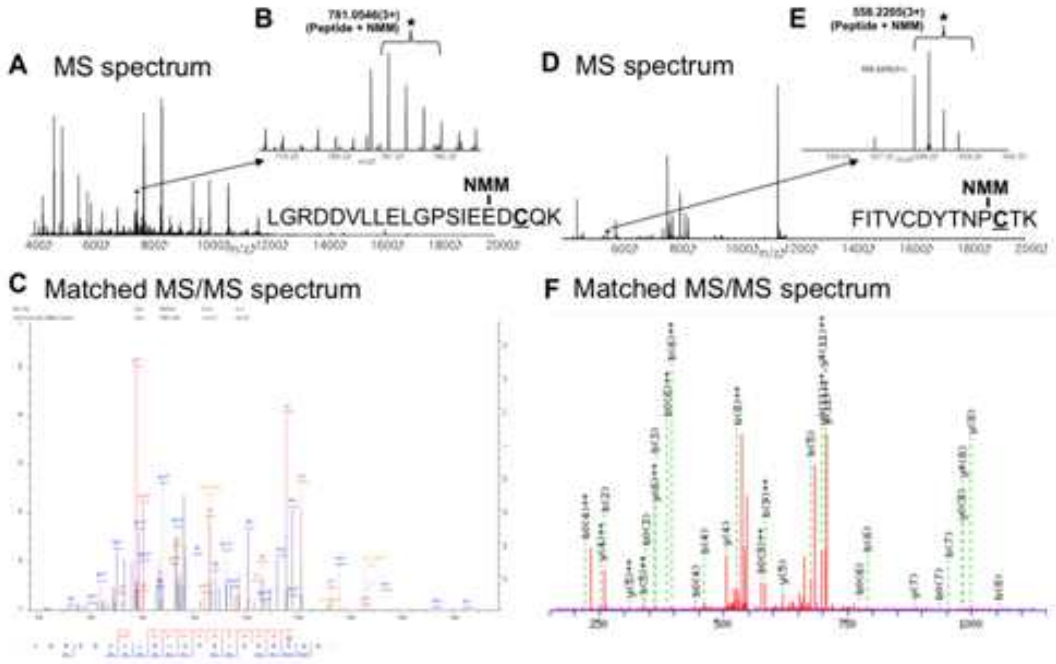


Figure 2. LC-MS/MS result shows MyD88 is palmitoylated at C113 and C274.

The trypsin-digested peptide samples were analyzed using an LC-MS/MS system consisting of a Nano Acquity UPLC system (Waters, USA) and an LTQ Orbitrap Elite mass spectrometer (Thermo Scientific, USA) equipped with a nano-electrospray source. Low-confident spectra were obtained using a 7T LTQ FT mass spectrometer (Thermo Scientific, USA). The peptides were separated over 90 min on a homemade microcapillary C18 column (100 mm i.d. x 200 mm length, particle size 3 mm, 124 Å), then the MS (A and D) and MS/MS (C and F) spectra were acquired over the entire run time. Raw data were processed using MaxQuant v1.5.2.8 or MASCOT searching tools. Expanded spectra (B and E) show the ions of the NMM-modified peptides,

and the peptides were identified by the matched MS/MS spectra (C and F) based on b- and y-fragment ions series.

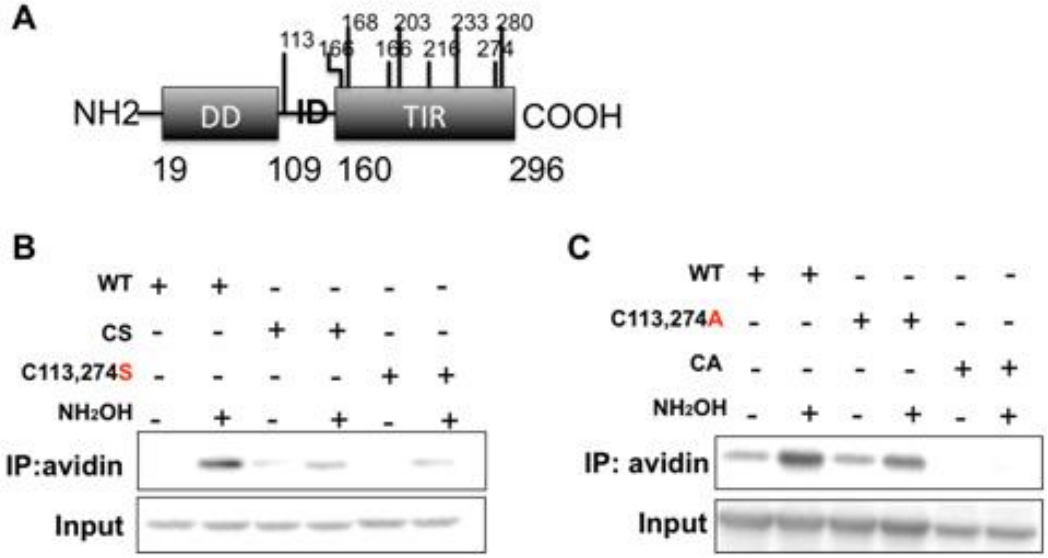


Figure 3. MyD88 is palmitoylated at C113 and C274.

A) A, Schematic presentation of MyD88 and its binding domains. The Death Domain (DD) accounts for MyD88 homodimerization. The Intermediate Domain (ID) contains IRAK4 binding motif and the palmitoylated site C113. The TIR domain contains eight remaining cysteine residues including palmitoylated sites C274 and is responsible for TLR4 binding-dependent signal transduction. B and C) Acyl-biotin exchange assay revealed by western blotting. HEK293T cells were transfected with wild-type and mutant MyD88-GyrB (n=10). CS or CA indicates MyD88 mutant where all 9 Cys are mutated to serine or alanine, respectively.

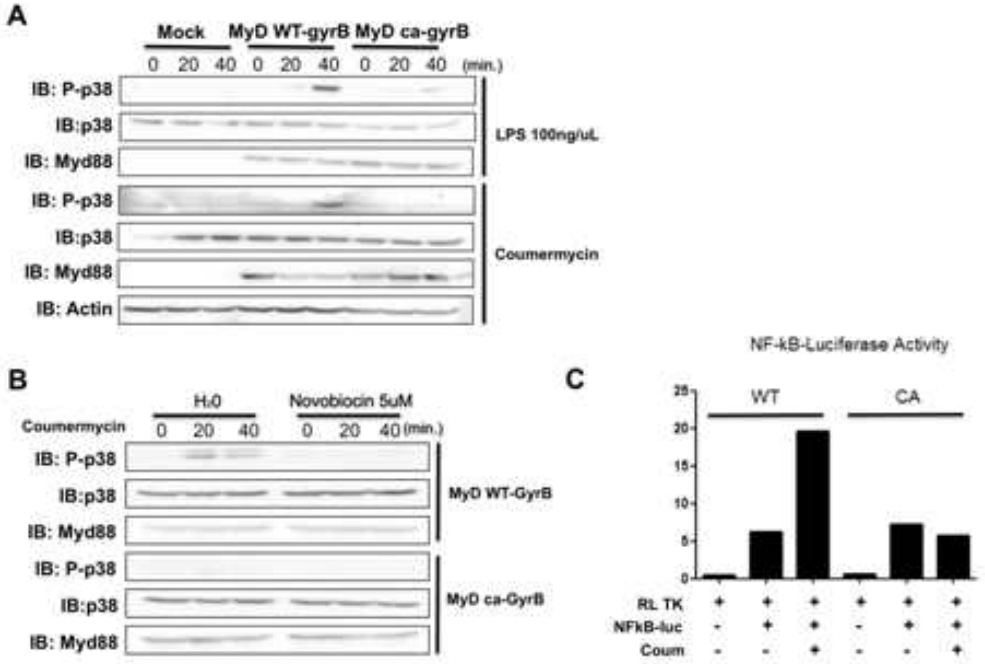


Figure4. Palmitoylation of MyD88 mediates P-p38 and NF-kB activation activation.

A) Western blot analysis. 0.1ug/mL LPS was treated in HEK293T cells stably expressing wild-type or mutant MyD88-GyrB in a time dependently as illustrated (0, 20, 40 minutes). HEK293T cells that are not transfected with MyD88-GyrB are indicated as Mock. B) Western blot analysis. 5uM novobiocin was pre-treated for 3 h before treatment of 0.1uM of coumermycin A1 in HEK293T cells stably expressing wild-type or mutant MyD88-GyrB. Ultra-pure distilled water was used as a negative control for pre-treatment of novobiocin. C) NF-kB luciferase activity revealed by bar graph using GraphPad Prism 5. HEK293T cells stably expressing wild-type or mutant MyD88-GyrB were transiently transfected with NF-kB luciferase

plasmid and pRL TK control plasmid. pRL-TK-only groups were used as negative control. After 30 h incubation, cells were treated with 0.1uM coumermycin A1 for 20 min followed by luciferase activity measurement.

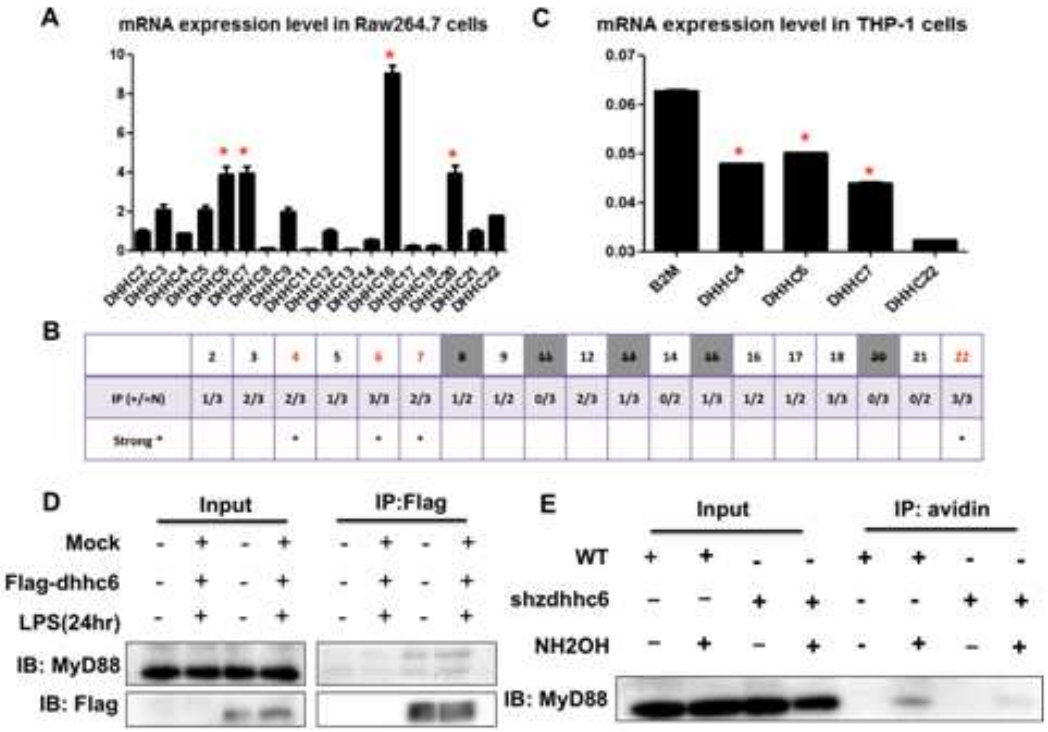


Figure 5. Screening MyD88-specific DHHC protein.

A) A realtime quantitative RT-PCR result using each DHHC specific primer in Raw 264.7 cells. B) Table summarizing the number of binding of MyD88 occurred with each DHHC after they were co-transfected in HEK293T cells followed by flag immuoprecipitation (IP=immunoprecipitation, + = the number of positive binding, N = total number of trial). Starred if the binding was strong. C) a realtime quantitative RT=PCR result in THP-1 cells. D) Flag-DHHC6 was transiently over expressed in HEK293T cells followed by flag immunoprecipitation using anti-flag affinity gel. Empty CAG vector (Mock) was used as a negative control. 0.1ug/mL LPS was treated for 24 h before harvest. E) Acyl-biotin exchange assay revealed by western

blotting. Shdhhc6 was stably expressed in raw 264.7 cells and were subject to ABE analysis. Non-transfected raw 264.7 cells were used as a negative control. Anti-MyD88 antibody was used for immunoblotting.

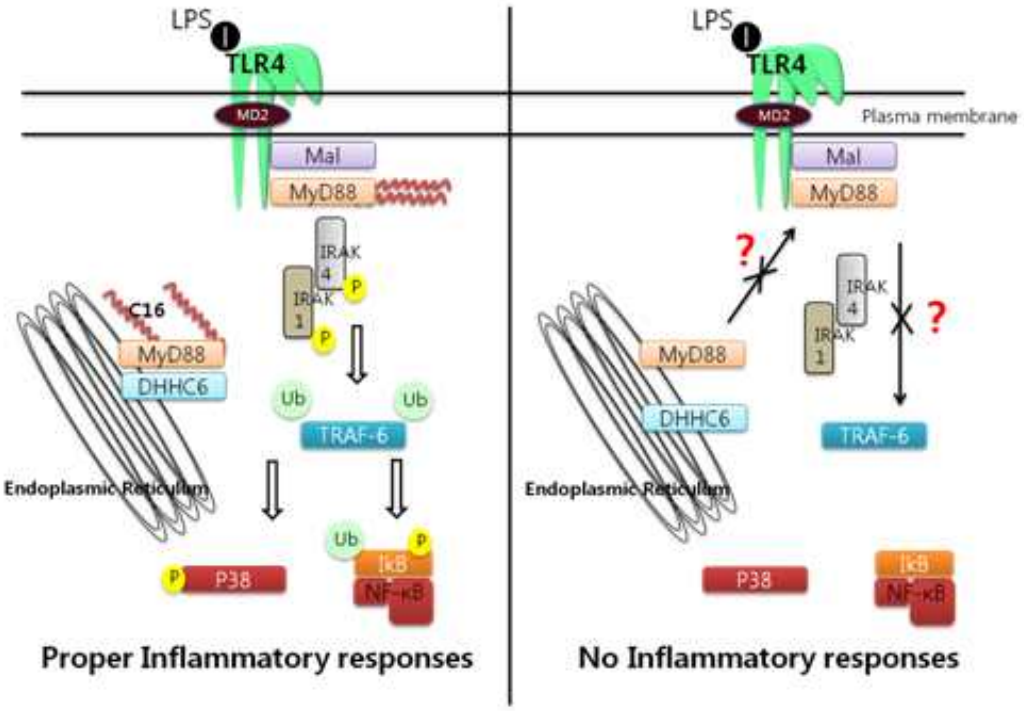


Figure 6. Schematic Drawing of MyD88 palmitoylation event in macrophage

A schematic drawing illustrating the event of MyD88 palmitoylation in macrophages.

Reference

(1) Bijlmakers MJ, Marsh M (2003) The on-off story of protein palmitoylation. Trends Cell Biol 13(1):32 - 42.

(2) Roth AF, et al. (2006) Global analysis of protein palmitoylation in yeast. Cell 125(5): 1003 - 1013.

(3) Jia L, et al. (2014) A mechanism regulating G protein-coupled receptor signaling that requires cycles of protein palmitoylation and depalmitoylation. J Biol Chem 289(9): 6249 - 6257.

(4) Bekirov IH, Nagy V, Svoronos A, Huntley GW, Benson DL. 2008. Cadherin-8 and N-cadherin differentially regulate pre- and postsynaptic development of the hippocampal mossy fiber pathway. Hippocampus 18:349 - 363

(5) Benson DL, Tanaka H. 1998. N-cadherin redistribution during synaptogenesis in hippocampal neurons. J Neurosci 18:6892 - 6904.

(6) Bamji SX, Shimazu K, Kimes N, Huelsken J, Birchmeier W, Lu B, Reichardt LF. 2003. Role of beta-catenin in synaptic vesicle localization and presynaptic assembly. Neuron 40:719 - 731.

(7) Hang, H.C. et al. Chemical probes for the rapid detection of fatty-acylated proteins in mammalian cells. J. Am. Chem. Soc. 129, 2744 - 2745 (2007)

(8) Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. Nat. Immunol. 5987-995

(9) T. Horng, G.M. Barton, R. Medzhitov TIRAP: an adapter

molecule in the Toll signaling pathway Nat. Immunol., 2 (2001), pp. 835 - 841

(10) Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol. 2007; 2:675 - 680.

(11) Medzhitov R, Kagan JC. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell.2006; 125: 943 - 55. pmid:16751103 doi: 10.1016/j.cell.2006.03.047

(12) Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. Nat. Rev. Immunol. 4, 499 - 511.

(13) Fitzgerald, K.A., Rowe, D.C., Barnes, B.J., Caffrey, D.R., Visintin, A., Latz, E., Monks, B., Pitha, P.M., and Golenbock, D.T. (2003). LPSTLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J. Exp. Med. 198, 1043 - 1055

(14) Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S.O., Goode, J., Lin, P., Mann, N., Mudd, S., et al. (2003). Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. Nature 424, 743 - 748.

(15) Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T., and Seya, T. (2003a). TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat. Immunol. 4, 161 - 167.

(16) Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003a). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 301, 640 - 643.

(17) Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., and Akira, S. (2003b). TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat. Immunol. 4, 1144 - 1150.

(18) Fukata Y, Bredt DS, Fukata M. Protein Palmitoylation by DHHC Protein Family. CRC Press; 2006. Chapter 5. Frontiers in Neuroscience.

(19) Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell.2006;124:783 - 801. pmid:16497588 doi: 10.1016/j.cell.2006.02.015

(20) Fukata, Y. & Fukata, M. Protein palmitoylation in neuronal development and synaptic plasticity. Nat. Rev. Neurosci. 11, 161 - 175 (2010).

(21) Ohno, Y. et al. Analysis of substrate specificity of human DHHC protein acyltransferases using a yeast expression system. Mol. Biol. Cell 23, 4543 - 4551 (2012)

(22) Into T, Inomata M, Nakashima M, et al. (2008) Regulation of MyD88-dependent signaling events by S nitrosylation retards toll-like receptor signal transduction and initiation of acute-phase immune responses. Mol Cell Biol 28, 1338 - 1347

(23) Häcker H., Redecke V., Blagoev B., Kratchmarova I., Hsu L. C., Wang G. G., Kamps M. P., Raz E., Wagner H., Häcker G., Mann M., Karin M. (2006) Nature 439, 204 - 207

(24) Burns, K., S. Janssens, B. Brissoni, N. Olivos, R. Beyaert, and J. Tschopp. 2003. Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due

to its failure to recruit IRAK-4. J. Exp. Med. 197: 263 - 268

(25) Mendoza-Barbera´, E., M. A. Corral-Rodrı´guez, A. Soares-Schanoski, M. Velarde, S. Macieira, A. Messerschmidt, E. Lo´pez-Collazo, and P. Fuentes-Prior. 2009.

(26) Gorleku OA, Barns AM, Prescott GR, Greaves J, Chamberlain LH (2011) Endoplasmic reticulum localization of DHHC palmitoyltransferases mediated by lysine-based sorting signals. J Biol Chem 286(45):39573 - 39584.

(27) Gregory J. Fredericks, FuKun W. Hoffmann, Aaron H. Rose, Hanna J. Osterheld, Franz M. Hess, Frederic Mercier, and Peter R. Hoffmann. Stable expression and function of the inositol 1,4,5-triphosphate receptor requires palmitoylation by a DHHC6/selenoprotein K complex. PNAS 2014 111: 16478-16483.

(28) Jong-Seok Moon, Seonmin Lee, Mi-Ae Park, Ilias I. Siempos, Maria Haslip, Patty J. Lee, Mijin Yun, Chun K. Kim, Judie Howrylak, Stefan W. Ryter, Kiichi Nakahira and Augustine M.K. Choi (2015) UCP2-induced fatty acid synthase promotes NLRP3 inflammasome activation during sepsis J Clin Invest; 125(2): 665-680

(29) Van der Heide, J. J., Biol, H. J., Donker, J. M., Wilmink, J. M., and Tegzess, A. M. (1993) N. Engl. J. Med. 329, 769 - 773

(30) Belluzzi, A., Brignola, C., Campieri, M., Pera, A., Boschi, S., and Miglioli, M. (1996) N. Engl. J. Med. 334, 1557 - 1560 34.

(31) Cappelli, P., DiLiberato, L., Stuard, S., Ballone, E., and Albertazzi, A. (1997) J. Nephrol. 10, 157 - 162 35.

(32) Cleland, L. G., French, J. K., Betts, W. H., Murphy, G. A., and Elliott, M. J. (1988) J. Rheumatol. 15, 1171 - 1475 36.

(33) Kremer, J. M., Jubiz, W., and Michalek, A. E. A. (1987) Annu. Int. Med. 106, 497 - 504

(34) Farrar M. A., Alberol-Ila, Perlmutter R. M. (1996) Nature 383, 178 - 181

(35) Mitchell DA, Vasudevan A, Linder ME, Deschenes RJ (2006) Protein palmitoylation by a family of DHHC protein S-acyltransferases. J Lipid Res 47(6):1118 - 1127.

(36) Antinone, S. E., Ghadge, G. D., Lam, T. T., Wang, L., Roos, R. P., and Green, W. N. (2013). Palmitoylation of superoxide dismutase 1 (SOD-1) is increased for familial amyotrophic lateral sclerosis-linked SOD-1 mutants. J. Biol. Chem. 288, 21606 - 21617. doi:10.1074/jbc.M113.487231

(37) Loiarro, M., G. Gallo, N. Fanto', R. De Santis, P. Carminati, V. Ruggiero, and C. Sette. 2009. Identification of critical residues of the MyD88 death domain involved in the recruitment of downstream kinases. J. Biol. Chem. 284: 28093 - 28103

(38) Medzhitov, R., P. Preston-Hurlburt, E. Kopp, A. Stadlen, C. Chen, S. Ghosh, and C. A. Janeway Jr. 1998. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol. Cell 2: 253 - 258

국문초록

MyD88 팔미토일화 반응에 따른 대식세포 염증반응에 관한 연구

점차적으로 더 많은 단백질들이 팔미토일화 된다고 밝혀지고 있지만 팔미토일화 반응과 TLR4 신호체계를 통한 염증반응의 관계에 대한 연구는 알려진 바가 없다. 본 석사 학위 논문에는 myeloid differentiation primary response protein (MyD88) 이 팔미토일화 된다는 사실을 acyl-biotin exchange, click chemistry, 그리고 cysteine mutagenesis 를 이용하여 다양하게 증명하였고 총 9개의 cysteine residue 중에 1번 (C113) 과 8번 (C274) 이 팔미토일화 된다는 것을 mass spectrometry 를 통해 검증했다. 또한 MyD88 의 9개 cysteine 기를 모두 alanine 혹은 serine 으로 치환했을 때 P-p38 과 NFkB 와 같은 TLR4 신호체계에 속한 단백질들의 기능이 저하됨을 발견함으로서 MyD88 의 팔미토일화 반응이 TLR4 염증체계에 필수적이라는 결론을 내었다.

더하여 MyD88을 팔미토일화 시키는 palmitoyl transferase 를 찾기 위해 RT-PCR, short-hairpin RNA, immunoprecipation, acyl-biotin exchange 와 같은 실험을 실시하여 23개의 aspartate-histidine-histidine-cysteine (DHHC) motif를 가지고 있는 palmitoyl transferase 단백질 중에 DHHC6이 잠정적으로 MyD88의 팔미토일화 반응에 참여 할 것 이라는 결론을 내었다. 끝으로 대식세포에서 MyD88 팔미토일화 반응은 TLR4 신호체계를 통한 염증반

응에 중요하다는 메시지를 담고 있다고 본다.

주요어: 팔미토일화 반응, 염증반응, 대식세포, TLR4 신호체계

학번: 2013-24025