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Biochemical Isolation of the Myddosome from Murine Macrophages

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Abstract

Ligand-induced macromolecular protein complex formation has emerged as a common means by which the innate immune system activates signal transduction pathways essential for host defense. Despite their structural divergence, key signaling molecules in diverse innate immune pathways mediate signal transduction by assembling higher-order protein complexes at specific subcellular locations in a stimulus-dependent manner. These protein complexes are collectively known as the supramolecular organizing centers (SMOCs), which link active receptors to a variety of downstream cellular responses. In the Toll-like receptor (TLR) pathway, the signaling adaptor MyD88 is the core of a SMOC called the myddosome, which is composed of the sorting adaptor TIRAP and the IRAK family kinases. Depending on the microbial ligands encountered, the myddosome can be assembled at the plasma membrane or endosomes, thereby leading to NF- κ B and AP-1 activation, and the subsequent expression of pro-inflammatory cytokines. Herein, we provide a detailed protocol for studying myddosome assembly in murine bone marrow-derived macrophages (BMDMs).

Keywords

TLR; SMOCs; MyD88; Myddosome; Immunoprecipitation

1 Introduction

The innate immune system is crucial for protecting the host from infections and maintaining homeostasis [1, 2]. At the cellular level, the innate immune system is composed of a diverse array of host proteins called pattern recognition receptors (PRRs) [1]. These receptors serve as sentinels at the cell surface, intracellular compartments, and the cytosolic space to detect conserved structural components or virulence activities associated with microorganisms [3, 4]. Upon sensing their cognate ligands, PRRs recruit downstream adaptor proteins to propagate signal transduction cascades that activate a broad spectrum of host responses to contain infections and promote adaptive immune responses [1, 3].

Among the PRR families, the Toll-like receptors (TLRs) are best-understood. Genetic and biophysical analysis dating to the late 1990s (and continuing today) revealed the microbial ligands sensed by TLRs, the structures of TLRs, dozens of factors that regulate TLR signaling, and subsequent transcriptional responses [1]. This increased knowledge of the

ligands and host factors that promote TLR signaling has revealed this protein family as an ideal model to elucidate the organizing principles that explain the natural operation of the innate immune system.

The mouse and human genomes collectively encode 13 TLRs, but neither human nor mouse cells express all of the 13 TLRs [1]. Human cells express TLRs 1–10; while mouse cells express TLRs 1–9 and TLRs 11–13. Based on their subcellular distribution, TLRs are categorized into cell surface or endosomal receptors [1]. Specifically, TLRs 1, 2, 4, 5, and 6, and 10 localize at the cell surface, and recognize surface molecules derived from microorganisms. In contrast, TLRs 3, 7–9, and 11–13 localize within the endosomal network where they sense nucleic acids and components of intracellular parasites [1, 3]. In summary, the steady-state localization of TLRs correlates with the nature of the microbial ligands they detect, thereby maximizing the efficiency for ligand recognition. Representative TLR ligands are listed in Table 1. Structurally, TLRs are type I transmembrane proteins featuring three distinct domains: a leucine-rich repeat (LRR) containing ectodomain, a transmembrane domain, and a cytosolic tail harboring the Toll/IL-1 receptor homology (TIR) domain [5]. Each of these domains exerts unique molecular functions, with the LRR domain sensing microbial ligands and interacting with accessory proteins, the transmembrane domain maintaining the membrane topology of TLRs, and the TIR domain recruiting downstream proteins to trigger signal transduction cascades [1].

TLRs themselves do not possess enzymatic functions, yet they induce enzyme-dependent signaling cascades upon microbial detection. To promote signal transduction, the TIR domain of TLRs recruits TIR domain-containing cytosolic adaptor proteins via TIR-TIR interactions upon ligand sensing [1]. MyD88, TIRAP (also known as MAL), TRAM, and TRIF are four well-characterized TIR-domain-containing adaptors that link activated TLRs to downstream enzymes such as IRAK family kinases and TRAF family E3 ubiquitin ligases [6–10]. The signaling cascades mediated by these enzymes culminate in the activation of several transcriptional factors such as NF- κ B, AP-1, and interferon (IFN) regulatory factors (IRFs). These factors subsequently trigger transcriptional responses exemplified by the expression of pro-inflammatory cytokines, IFNs, and IFN-stimulated genes (ISGs) [1]. Specifically, the adaptors TIRAP and MyD88 induce the production of pro-inflammatory cytokines by activating the NF- κ B and AP-1-dependent signaling cascades [1, 11], whereas the adaptors TRAM and TRIF engage multiple kinases (i.e., TBK1, IKK ϵ , and RIP kinases) to promote IFN production and sustained NF- κ B activation [1, 12]. In summary, the utilization of distinct sets of TIR domain-containing adaptors ensures differential signaling outcomes after TLR activation [1].

Cell biological analyses have functionally categorized these TIR domain containing adaptors into sorting adaptor and signaling adaptor families [1]. TIRAP and TRAM define the sorting adaptor family, which determine the subcellular sites of TLR signal transduction by sensing ligand-bound TLRs [1]. Accordingly, MyD88 and TRIF define the signaling adaptor family as they are recruited by the sorting adaptors to the sites of signal transduction and further interact with downstream host proteins to execute diverse biological functions [1]. Of note, MyD88 serves as the signaling adaptor for all the TLRs except for TLR3, which exclusively uses TRIF as its signaling adaptor [1].

Originally identified as a signature gene of which the transcription is induced during myeloid cell differentiation triggered by IL-6 [13], MyD88 is composed of three domains: a death domain, a short linker domain, and a TIR domain [14]. This unique protein architecture determines the mechanism by which MyD88 propagates TLR signaling: its C-terminal TIR domain associates with the TIR domains of the sorting adaptor TIRAP and upstream TLRs, and the N-terminal death domain of MyD88 interacts with IRAK family kinases [15, 16]. As such, MyD88 links activated receptors to executors of signal transduction. In keeping with its crucial role in TLR signaling, somatic mutations of MyD88 have been implicated in a variety of host immune disorders. For instance, a gain-of-function mutation (e.g., L265P) of MyD88 induces the simultaneous activation of the NF- κ B pathway in the absence of TLR ligands, thereby promoting the oncogenesis of a subtype of aggressive B-cell lymphoma known as the activated B-cell-like diffuse large B-cell lymphoma (ABC-DLBCL) [17]. In contrast, loss- of- function MyD88 mutations (e.g., R196C, L93P) increase the susceptibility of patients to recurrent pyogenic bacterial infections [18]. Therefore, understanding the molecular mechanisms of MyD88-dependent signal transduction has significant biomedical implications.

Structural analysis has demonstrated that MyD88 is the core component of a macromolecular complex named the myddosome, in which the death domain of MyD88 oligomerizes with the death domains of IRAK2 and IRAK4 kinases [19, 20]. The formation of the myddosome increases the local concentration of IRAK family kinases significantly, thereby facilitating the dimerization and transphosphorylation of these kinases, which leads to NF- κ B activation [21]. Therefore, the structural insights on myddosome formation illustrate a signaling paradigm whereby the assembly of MyD88- IRAK4- IRAK2 proteins into a higher-order helical structure couples receptor activation to the induction of downstream host responses upon TLR activation [22, 23]. In this regard, the myddosome joins other macromolecular complexes in the innate immune system, such as the inflammasome and the RIG-I-MAVS complex, which are collectively referred to as the supramolecular organizing centers (SMOCs) [22].

Although in vitro structural analyses provide an elegant model for myddosome assembly, whether the myddosome is formed in living cells in response to microbial ligands or how the myddosome is assembled at distinct subcellular locations have remained elusive. Cell biological and biochemical characterization of TIRAP, the aforementioned sorting adaptor, demonstrated that the formation of the myddosome is a microbe-inducible process, and further revealed that TIRAP is the first-defined regulator of myddosome formation [24]. Moreover, these findings demonstrated that the diverse lipid-binding spectrum of TIRAP allows this sorting adaptor to mediate myddosome formation at the plasma membrane (e.g., TLR4 activation) and at endosomes (e.g., TLR9 activation) in response to cognate microbial ligands [24]. In conclusion, myddosome assembly occurs at multiple subcellular sites and is subject to delicate regulation. Herein, we describe detailed procedures to isolate and store primary bone marrow-derived macrophages (BMDMs). Furthermore, we outline a protocol for monitoring myddosome formation by immunoprecipitation. The protocol described here should also be applicable to tracking myddosome formation in other types of murine primary cells or cell lines.

2 Materials

2.1 Differentiation and Cryo-Storage of Primary BMDMs

1. Mice.
2. Autoclaved scissors and forceps.
3. Flushing medium: Phosphate-buffered Saline (PBS).
4. 10 mL syringes.
5. 26 G × 1/2 needles.
6. Sterile, non-tissue culture treated 10 cm petri dishes.
7. 70% Ethanol.
8. Paper towels.
9. Ice.
10. 2 Beakers (200–300 mL).
11. Sterile 50 mL tubes.
12. Sterile 5, 10, and 25 mL pipettes.
13. Hemocytometer.
14. Macrophage medium: RPMI supplemented with 10% fetal bovine serum (FBS), 100 U/mL Penicillin-Streptomycin, Glutamine (2 mM), Sodium Pyruvate (1 mM), and 20% L929-conditioned medium. Alternatively, use complete DMEM for **immortalized** BMDMs (iBMDMs): DMEM supplemented with 10% FBS, 100 U/mL Penicillin-Streptomycin, Glutamine (2 mM), Sodium Pyruvate (1 mM).
15. Centrifuge with rotor fitting 50 mL tubes.
16. Cell culture facility and equipment including 37 °C, 5% CO₂, water-jacketed cell culture incubator, and laminar flow hood.
17. Freezing medium: FBS with 10% DMSO (Dimethyl Sulfoxide, Sigma, D2650).
18. Cryo vials (1.5 mL volume).
19. Isopropanol freezing chamber (ThermoFisher, 5100–0001).
20. Glass Pasteur pipette.
21. Aspirator.

2.2 Thawing Frozen BMDMs from the Liquid Nitrogen Tank

1. Cryo vials containing BMDMs.
2. Dry Ice.
3. 37 °C water bath.

4. 70% Ethanol.
5. Sterile 50 mL tubes.
6. Sterile 5, 10, and 25 mL pipettes.
7. Sterile, non-tissue culture treated 10 cm petri dishes (for primary BMDMs). Alternatively, use treated petri dishes for iBMDMs.
8. Macrophage medium.
9. Tissue culture grade PBS.
10. Ice.
11. Centrifuge with rotor fitting 50 mL tubes.
12. Cell culture facility and equipment including a 37 °C, 5% CO₂, water-jacketed cell culture incubator, and a laminar flow hood.

2.3 Stimulating BMDMs with TLR Ligands to Induce Myddosome Formation

1. Sterile 50 mL tubes.
2. Sterile 5, 10, and 25 mL pipettes.
3. Sterile, non-tissue culture treated 10 cm petri dishes.
4. Macrophage medium for primary BMDMs. Alternatively, use complete DMEM for iBMDMs.
5. PBS-EDTA solution: 4 mM EDTA in PBS; (EDTA, ultra- pure, ThermoFisher, 15575020). PBS-EDTA is used to detach BMDMs from petri dishes without affecting the surface expression of TLRs and other TLR accessory molecules (*see* Note 3).
6. Ice.
7. Centrifuge with rotor fitting 50 mL tubes.
8. Cell culture facility and equipment including a 37 °C, 5% CO₂, water-jacketed cell culture incubator and laminar flow hood.
9. TLR Ligands:
 - TLR4: LPS from *E. coli* [Enzo Life Sciences, ALX-581-012-L002, Serotype O111:B4 (TLRgrade™)].
 - TLR2: Pam3CSK4 (Invivogen, vac-pms, VacchiGrade™).
 - TLR9: CpG [(T*C*C*A*T*G*A*CG*T*T*C*C*T*G*A*C*G*T*T*); synthesized from MWG Operon or IDT]. * stands for phosphorothioate linkage.

³.When handling primary and iBMDMs, the PBS-EDTA solution is a preferred reagent for cell detachment. It is advised to not use trypsin on BMDMs, as trypsin digestion will result in unwanted cleavage of PRRs expressed on the cell surface of BMDMs.

10. Hemocytometer.
11. Round-Bottom Polystyrene Tubes (Falcon® 5 mL Round Bottom Polystyrene Test Tube Product, #352054).

2.4 Isolation of the Myddosome from Stimulated BMDMs by Immuno-precipitation

1. Lysis/Wash buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% (v/v) glycerol, 2 mM DTT, and 1% (v/v) NP-40 (also known as IGEPAL® CA-630).
2. Safe-Lock 1.5 mL microcentrifuge (Eppendorf).
3. Complete mini EDTA-free protease inhibitor tablets (Sigma, 11836170001).
4. PhosSTOP™ Phosphatase inhibitor tablets (Sigma, 4906845001) (*see* Note 1).
5. Protein G Sepharose 4 Fast Flow beads (Sigma, GE17-0618-01).
6. Immunoprecipitation-grade primary antibody against mouse MyD88 (anti-MyD88, Goat Polyclonal, R&D, AF3109).
7. Table-top centrifuge at 4 °C.
8. Heat block set at 100 °C.
9. 1× SDS loading buffer.
10. p200 pipette.
11. Fine-end (1–200 µL) disposable gel-loading pipette tip (VWR 37001–152).
12. Nutator in a 4 °C cold room.

2.5 Analysis of Myddosome Formation by Western Blotting

2.5.1 Western Blotting Reagents

1. Nitrocellulose Membrane (Biorad, 1620115) or PVDF membrane.
2. General-purpose blotting paper or Whatman paper.
3. 1 Large measuring cylinder (1 L).
4. Plastic containers/vessels (e.g., the cover of a pipette tip box) for membrane blocking and antibody incubation after protein transfer.
5. Wet gel transfer system (e.g., Bio-Rad Protean) and an ice block which fits into the chamber.
6. 10× SDS Running Buffer: 250 mM Tris-HCl, 1.92 M Glycine, 1% SDS (w/v) in H₂O. For 1 L: 30.2 g Tris-HCl, 144 g Glycine, 10 g SDS and fill up to 1 L with H₂O.

¹Altogether, β Glycerophosphate disodium salt hydrate (working concentration: 25 mM), sodium fluoride (working concentration: 10 mM), and sodium orthovanadate (working concentration: 1 mM) are cost-effective alternatives to the commercial phosphatase inhibitor tablets. Adding phosphatase inhibitors to the cell washing step prior to lysis could better preserve ligand-induced protein phosphorylation.

7. Wet transfer buffer: 25 mM Tris-HCl, Glycine, 192 mM Glycine, 0.05% SDS (w/v), 20% Methanol (v/v)
8. Blocking buffer: 5% nonfat milk in PBST.
9. Tween-20 [20% solution (v/v) in H₂O] (*see* Note 2).
10. 10× PBS: For 1 L, add 80 g NaCl, 2 g KCl, 26.8 g Na₂HPO₄ · 7H₂O, 2.4 g KH₂PO₄ and fill to a final volume of 1 L with H₂O. Adjust pH to 7.4 with HCl.
11. PBST Solution: 100 mL 10× PBS, 900 mL H₂O, 500 µL 20% Tween.
12. Gel-loading tips (VWR® Gel Loading Pipet Tips 37001–152).

2.5.2 Antibodies and Imaging Tools

1. Primary antibodies for blotting myddosome components:
 - IRAK2 (anti-IRAK2, Rabbit polyclonal, Prosci, 3595).
 - IRAK4 (anti-IRAK4, Mouse monoclonal, clone 2H9, Abcam, ab119942).
 - TIRAP (anti-TIRAP, Rabbit monoclonal, clone D6M9Z, CST, 13077).
 - MyD88 (anti-MyD88, Goat Polyclonal, R&D, AF3109, or anti-MyD88, rabbit polyclonal, Rockland, 600-401-955). Please note that the MyD88 antibody from Rockland is only suitable for detecting MyD88 from whole cell lysates by western blotting, this antibody does not work for immunoprecipitation.
2. Secondary antibodies:
 - Peroxidase AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch, 111-035-003).
 - Peroxidase AffiniPure Goat Anti-Mouse IgG (Jackson ImmunoResearch, 115-035-003).
 - Peroxidase AffiniPure Donkey Anti-Goat IgG (Jackson ImmunoResearch, 705-035-147).
3. 50 mL conical tubes.
4. ECL Substrate kit (SuperSignal™ West Femto Chemiluminescent Substrate, ThermoFisher, 34096).
5. Plastic wrap for western blotting development (Fisherbrand™ Clear Plastic Wrap, 22-305-655, Fishersci).
6. Developing equipment of choice (i.e., Biorad ChemiDoc™ XRS+ System).

²-100% Tween-20 is extremely viscous, prepare a 20% Tween-20 (v/v in H₂O) stock solution to reduce pipetting error when preparing the PBST solution.

3 Methods

3.1 Isolation, Differentiation, and Storage of Primary BMDMs

Carry out all methods with approval from your local animal ethics committee.

1. Sterilize dissection tools by filling two beakers with 200 mL 70% ethanol. Place dissection forceps and scissors in one beaker and move both the beakers to laminar flow hood.
2. Fill syringes with 10 mL of flushing medium and attach the 26 G $\times$ 1/2 needles. Usually, one syringe with 10 mL flushing medium is sufficient to flush two bones (left and right femur) of one mouse.
3. Euthanize a mouse by CO₂ inhalation.
4. Lay the mouse on its back on a diaper and spray the mouse with 70% Ethanol. Transfer the mouse into the hood.
5. Using the forceps to hold one leg up by the ankle. Make a small incision with the scissors into the skin. Starting at this incision, tear the skin from the ankle to the abdomen area. Please be aware not to cut into the peritoneum.
6. Using the forceps to hold the foot up and further cut the initial incision (in **step 5**) around the ankle. Next, separate the skin from the leg until the whole leg is exposed.
7. Use the scissors to separate the hip bone so that the whole leg is released from the body. Please do not to cut the femur at this step. Place the whole leg in a 10 cm petri dish (sterile).
8. Repeat the steps (**steps 5–7**) with the other leg. Before dissecting the other leg, sterilize the used scissors and forceps by putting them into other beaker containing 70% ethanol. Then briefly dry the scissors and the forceps with a piece of paper towel.
9. Use the scissors and the forceps to remove the muscle tissue so that the bones and the knee joint are exposed. Cut the leg at the knee joint to separate the femur, transfer the femur into a new 10 cm dish. (Optional: tibia could be kept too)
10. To obtain the bone marrow, hold one bone in the middle with the forceps and cut open both the sides of the bone with scissors. Carefully insert a needle into the bone marrow cavity and flush the marrow out into a 15 mL tube with 5–6 mL of flushing medium per bone. Turn the bone around and flush out the residual bone marrow from the other side (~2.5–3 mL flushing medium for each side). Repeat this step with the other femur bone.
11. Centrifuge the marrow for 5 min at $400 \times g$, aspirate the supernatant using a glass Pasteur pipette attached to a vacuum system, and resuspend the pellet in a 5 mL macrophage medium with gentle pipetting.

12. Determine the number of bone marrow progenitor cells using a hemocytometer. Plate $0.5\text{--}1 \times 10^7$ cells into a non-tissue culture treated 10 cm petri dish containing 10 mL macrophage medium. Incubate at 37 °C for 3 days.
13. Feed the BMDMs: On day 3 add an additional 4 mL of macrophage medium directly into each petri dish without removing the original 10 mL medium, so that the total volume macrophage medium in a petri dish is approximately 15 mL. Incubate at 37 °C for a further 3–4 days.
14. Aspirate the medium containing non-adherent cells via a glass Pasteur pipette attached to a vacuum system and add 5 mL of 4 °C PBS-EDTA solution per petri dish (*see* Note 3). Leave the petri dish in the hood for 5–10 min. Alternatively, incubate the petri dish in a refrigerator at 4 °C for 5 min.
15. Gently pipette the BMDMs up and down with a 10 mL pipette fitted to a pipette aid. Transfer the cells to a 50 mL tube. Repeat this step to finish collecting cells from all petri dishes.
16. Determine the cell number by hemocytometer. Proceed to Subheading 3.3 for cell stimulation or proceed to **step 17** below for long-term storage.
17. Prepare a suspension of BMDMs at $1 \times 10^8/\text{mL}$ in FBS. Gently mix this suspension with equal volume of freezing medium to make a final cell suspension at 0.5×10^8 BMDMs/mL in FBS with 10% DMSO.
18. Prepare 1 mL aliquots of the BMDM suspension into the cryo vials and freeze overnight using an isopropanol freezing chamber in a –80 °C freezer. Alternatively, freeze the BMDM aliquots in –20 °C for 2 h, then transfer them to a –80 °C freezer for overnight storage.
19. Transfer the cryo vials to a liquid nitrogen tank for long-term storage. Alternatively, if using iBMDMs proceed directly to Subheading 3.3.

3.2 Thawing BMDMs Stored in Liquid Nitrogen

1. Take out a cryo vial containing frozen BMDMs from the liquid nitrogen tank.
2. Open a 50 mL centrifuge tube and add 3 mL of 70% ethanol. Put the cryo vial in the 50 mL centrifuge tube containing 3 mL 70% ethanol. Place the 50 mL centrifuge tube in a 37 °C water bath and wait until the cells are thawed. Once the cells are thawed, take out the cryo vial, spray it with 70% ethanol, dry it with a piece of paper towel, and move it to a laminar flow hood.
3. Transfer the BMDMs into a 15 mL tube containing 4 mL macrophage medium.
4. Pellet the BMDMs by centrifugation at $400 \times g$ for 5 min at 4 °C. Aspirate the supernatant and add 2 mL of macrophage medium to resuspend the cell pellet.
5. Transfer the BMDMs into a petri dish containing 10 mL macrophage medium and incubate them overnight at 37 °C.
6. Check the BMDMs under a microscope. Most of the BMDMs should be adherent and alive. Please note that frozen iBMDMs can be thawed following the

same steps listed above. When thawing iBMDMs, use complete DMEM medium instead of macrophage medium.

3.3 Cell Stimulation and Myddosome Isolation by Immuno-precipitation

This method is suitable for analyzing myddosome formation from primary BMDMs and iBMDMs. (See Fig. 1 for the experimental flowchart.)

1. Move the petri dishes containing BMDMs into the laminar flow hood. Aspirate the medium, and gently add 5 mL of PBS- EDTA solution per dish (*see* Note 3), and incubate for 5 min in the hood.
2. Gently pipette the BMDMs up and down with a 10 mL pipette fitted to a pipette-aid. Transfer BMDMs to a 50 mL tube. Repeat this step to finish collecting BMDMs from all the petri dishes.
3. Determine the cell number using a hemocytometer. In general, one petri dish can yield $\sim 1.5\text{--}3 \times 10^7$ BMDMs.
4. Adjust the concentration of primary BMDMs to $1 \times 10^6/\text{mL}$ with macrophage medium (use complete DMEM for iBMDMs) (*see* Note 4). Distribute 1 mL of cell suspension (1×10^6 cells) to a 5 mL round-bottom polystyrene tube (*see* Note 5). Loosely close the lid. The total number of tubes required for each experiment should be determined according to the number of time points examined. For instance, when examining myddosome formation induced by LPS at three different time points (i.e., 30, 60, 120 min), prepare four round-bottom polystyrene tubes (one tube per time point plus an extra tube for untreated control) with each tube containing 1×10^6 BMDMs in 1 mL medium with proper labeling.
5. Stimulate cells with TLR ligands in **reverse** chronological order: Add the TLR ligand of choice to the cells that will be stimulated for the longest time period first. In the case of LPS stimulation, add LPS to a final concentration of $1 \mu\text{g}/\text{mL}$ to the cells that will be stimulated for 120 min. Second, set up a timer for 120 min (*see* Note 6 for the working concentration of TLR2 and TLR9 ligands). Third, as the timer counts down, stimulate the remaining tubes of cells with LPS at indicated time points (i.e., 60, 30 min). Lastly, at the end of the 120 min time course, add 3 mL of ice-cold PBS containing phosphatase inhibitors directly to each tube, spin the cells for 5 min at $400 \times g$ at 4°C to pellet the cells. This procedure synchronizes the collection of cells stimulated at different time points.

⁴. Up to 1×10^8 BMDMs can be resuspended in 1 mL medium in a tube. A similar procedure has been used to stimulate cells to characterize the Death-Inducing Signaling Complex and the TNFR1 signaling complex [25, 26].

⁵. Once activated by TLR ligands, BMDMs become significantly more adherent. Thus, it is recommended to stimulate cells in a round-bottom FACs tube, which ensures maximum and efficient cell recovery after stimulation. Otherwise, if BMDMs are stimulated in petri dishes, it is difficult to collect all the cells from the petri dishes efficiently at the end point of an experiment even with the aid of a rubber policeman/cell scraper.

⁶. The optimal working concentration of Pam3CSK4 is $0.5\text{--}1 \mu\text{g}/\text{mL}$; the optimal working concentration of CpG is $5 \mu\text{M}$.

6. Aspirate the supernatant, wash the cell pellets one more time with 3 mL of ice-cold PBS containing phosphatase inhibitors. Spin the cells for 5 min at $400 \times g$ at 4 °C.
7. Aspirate the supernatant, place the tubes on ice, add 500 µL of lysis buffer with protease inhibitors and phosphatase inhibitors. Resuspend the cell pellets by gently pipetting 2–3 times with an adjustable pipette (P1000) mounted with a disposable pipette tip. Change the tips when resuspending different samples.
8. Keep the tubes on ice and let the cells incubate with lysis buffer for 15–30 min. At the end of the incubation, transfer the lysates to 1.5 mL microcentrifuge tubes.
9. Clear the lysates by centrifuging at $16,000 \times g$ (or top speed) for 15 min at 4 °C in a table-top centrifuge.
10. Transfer the cleared lysates to a new set of microcentrifuge tubes using an adjustable (P1000) pipette fitted with a disposable tip. Do not disturb the pellets; leave the last 20 µL of cleared lysates/supernatants in the old set of microcentrifuge tubes. Keep the cleared lysates on ice.
11. Transfer 50 µL of the cleared lysates (~1/10 of total cell lysates) to a new set of microcentrifuge tubes. This set of samples will serve as the inputs for western blotting analysis. Add 15 µL of 5× SDS loading buffer to the input samples. Gently mix the loading buffer and input samples by pipetting, and boil samples at 100 °C for 5 min. Store the boiled input samples at –20 °C.
12. Add 1 µg of the MyD88 antibody to the remaining cleared lysates (~400 µL) (*see* Note 7).
13. Prepare protein G Sepharose beads for isolating the myddosome from the cleared lysates by immunoprecipitation (*see* Note 8). In general, 15 µL (bead volume or 30 µL 50% slurry) of the protein G Sepharose beads is sufficient for isolating the myddosome from one sample. To prepare the desired volume of beads for four immunoprecipitation reactions, equilibrate protein G beads by washing 120 µL of protein G sepharose slurry (beads: 20% ethanol = 1:1) with 3 mL of PBS, pellet the beads by centrifuging at $400 \times g$ for 3 min. Aspirate PBS, then resuspend the beads with 450 µL of lysis buffer. Add 100 µL of lysis buffer-beads mixture to the cleared lysates mix containing 1 µg anti-MyD88 antibody (from **step 12** of Subheading 3.3).
14. Place the microcentrifuge tubes on a nutator in a 4 °C cold room, incubate the tubes with gentle rotation for at least 2 h to immunoprecipitate the myddosome from the lysates.

⁷To reduce potential pipetting errors (due to a small pipetting volume), prepare an antibody mastermix solution by adding 4 µL of the MyD88 (1 µg/µL) antibody to a microcentrifuge tube containing 450 µL lysis buffer. Then equally distribute 100 µL of the antibody mastermix to the each of the four microcentrifuge tubes containing the cleared lysates.

⁸When pipetting the protein G beads, first fit a disposable tip to a P1000 pipette, then cut the end of the disposable tip with the scissors to create a wider opening at the end. This procedure reduces suction and ensures more accurate transfer of the beads.

15. At the end of the immunoprecipitation (*see* Note 9), pellet the beads by spinning at $3000 \times g$ for 30 s at 4 °C in a table-top centrifuge. Carefully aspirate the supernatant with a glass Pasteur pipette attached to a vacuum device, and leave 10–20 μL of remaining volume above the pellet.
16. Wash the beads with 500 μL of wash buffer (ice-cold lysis buffer), invert the tubes 3–5 times during each wash. Centrifuge the tubes at $3000 \times g$ for 30 s at 4 °C in a table-top centrifuge. Aspirate the washing buffer. Repeat the washing step two additional times, so that a total of three washes will be performed. After the last wash, centrifuge again to bring down any residual drops of wash buffer on the side of the tube.
17. To remove the residual wash buffer, fit a fine-end (1–200 μL) disposable gel-loading pipette tip (VWR 37001–152) to a p200 pipette, firmly push the end of the pipette tip to the bottom of a microcentrifuge tube containing the pelleted beads. Then, slowly release the push button of the pipette to aspirate the residual volume. The pelleted beads should turn white at this moment. Slowly pull the fine-end pipette tip out from the beads, be careful not to dislodge the beads.
18. Add 50 μL of 1 $\times$ SDS loading buffer to the beads, elute the bound myddosome complex by boiling the samples for 5 min on a heat block set at 100 °C. Alternatively, heat the samples for 15–20 min on a heat block set at 65 °C.

3.4 Analyzing Myddosome Formation by Western Blotting

1. Separate protein components in the myddosome eluates and input samples by SDS-PAGE. To one well/lane of a 10% SDS- PAGE gel, load 25 μL (1/2) of the myddosome eluates or 10 μL of the input samples (~30–50 μg of total cellular protein).
2. Perform electrophoresis at 130 V (constant voltage) for ~90 min.
3. Transfer the proteins from the gel to a nitrocellulose or PVDF (*see* Note 10) membrane using the wet transfer method (60 min at 100 V for BioRad MiniProtein gels).
4. Block the membrane in a plastic vessel for 1 h in blocking buffer at room temperature with constant shaking.
5. Add the primary antibody of choice at an appropriate concentration in PBST (*see* Note 11). Incubate overnight at 4 °C with constant shaking.

⁹The length of the incubation period of the myddosome immunoprecipitation is flexible. The formation of the myddosome could be detected as short as 2 h by incubating the MyD88 antibody and the protein G beads with the cleared lysates. On the other hand, overnight incubation of the antibody and the lysates could increase the signal-to-noise ratio of myddosome detection by western blotting.

¹⁰Nitrocellulose membrane and PVDF membrane are essentially interchangeable in terms of protein transfer. Please keep in mind that PVDF needs to be activated by methanol prior to setting up the protein transfer.

¹¹Optimal dilution for primary antibodies used to detect myddosome formation by western blotting: Anti-MyD88 (1:1000); Anti-IRAK2 (1:4000); Anti-IRAK4 (1:1000); Anti-TIRAP (1:1000).

6. Recycle the primary antibody and store it at 4 °C for repeated use. Wash the membrane with two quick washes (10–15 s) with PBST and three additional 5 min washes with PBST.
7. Add the peroxidase-conjugated secondary antibody of choice at 1:5000 in blocking buffer and incubate for 1 h at room temperature, with constant shaking.
8. Remove secondary antibody: Wash membranes with at least 3 × 5 min washes with PBST.
9. Briefly dry the membrane by tapping it on an edge of the plastic vessel and transfer the membrane on a plastic wrap or a parafilm extended on a flat surface. Add 1 mL of ECL Western blot substrate on the top of the plastic wrap/parafilm, incubate the membrane with the ECL solution (with the protein side toward the ECL solution) for 5 min at room temperature.
10. Tap to dry the membrane, transfer the membrane back to the vessel, and develop the blot using the developing equipment of choice.
11. To re-probe the blot with different antibodies, strip the membrane with a stripping solution as per the manufacturer's instructions. After stripping, re-block the membrane in a plastic vessel for 1 h in blocking buffer at room temperature with constant shaking.
12. Repeat **steps 4–10** with different antibodies (*see* Fig. 2 for a representative western blotting result of myddosome assembly upon LPS stimulation in iBMDMs).

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References

1. Brubaker SW et al. (2015) Innate immune pattern recognition: a cell biological perspective. *Annu Rev Immunol* 33:257–290 [PubMed: 25581309]
2. Chu H, Mazmanian SK (2013) Innate immune recognition of the microbiota promotes host-microbial symbiosis. *Nat Immunol* 14(7):668–675 [PubMed: 23778794]
3. Takeuchi O, Akira S (2010) Pattern recognition receptors and inflammation. *Cell* 140(6):805–820 [PubMed: 20303872]
4. Vance RE, Isberg RR, Portnoy DA (2009) Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. *Cell Host Microbe* 6(1):10–21 [PubMed: 19616762]
5. Kawai T, Akira S (2010) The role of pattern- recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* 11(5):373–384 [PubMed: 20404851]
6. Horng T et al. (2002) The adaptor molecule TIRAP provides signalling specificity for Toll- like receptors. *Nature* 420(6913):329–333 [PubMed: 12447442]
7. Yamamoto M et al. (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301(5633):640–643 [PubMed: 12855817]

8. Yamamoto M et al. (2002) Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* 420(6913):324–329 [PubMed: 12447441]
9. Yamamoto M et al. (2003) TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol* 4(11):1144–1150 [PubMed: 14556004]
10. Medzhitov R et al. (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell* 2(2):253–258 [PubMed: 9734363]
11. Kagan JC, Medzhitov R (2006) Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. *Cell* 125(5):943–955 [PubMed: 16751103]
12. Fitzgerald KA et al. (2003) IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 4(5):491–496 [PubMed: 12692549]
13. Lord KA, Hoffman-Liebermann B, Liebermann DA (1990) Nucleotide sequence and expression of a cDNA encoding MyD88, a novel myeloid differentiation primary response gene induced by IL6. *Oncogene* 5(7):1095–1097 [PubMed: 2374694]
14. Bonnert TP et al. (1997) The cloning and characterization of human MyD88: a member of an IL-1 receptor related family. *FEBS Lett* 402(1):81–84 [PubMed: 9013863]
15. Wesche H et al. (1997) MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* 7(6):837–847 [PubMed: 9430229]
16. Muzio M et al. (1997) IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* 278(5343):1612–1615 [PubMed: 9374458]
17. Ngo VN et al. (2011) Oncogenically active MYD88 mutations in human lymphoma. *Nature* 470(7332):115–119 [PubMed: 21179087]
18. von Bernuth H et al. (2008) Pyogenic bacterial infections in humans with MyD88 deficiency. *Science* 321(5889):691–696 [PubMed: 18669862]
19. Lin SC, Lo YC, Wu H (2010) Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature* 465(7300):885–890 [PubMed: 20485341]
20. George J et al. (2011) Two human MYD88 variants, S34Y and R98C, interfere with MyD88-IRAK4-myddosome assembly. *J Biol Chem* 286(2):1341–1353 [PubMed: 20966070]
21. Ferrao R et al. (2014) IRAK4 dimerization and trans-autophosphorylation are induced by Myddosome assembly. *Mol Cell* 55(6):891–903 [PubMed: 25201411]
22. Kagan JC, Magupalli VG, Wu H (2014) SMOCs: supramolecular organizing centres that control innate immunity. *Nat Rev Immunol* 14(12):821–826 [PubMed: 25359439]
23. Gay NJ, Gangloff M, O'Neill LA (2011) What the Myddosome structure tells us about the initiation of innate immunity. *Trends Immunol* 32(3):104–109 [PubMed: 21269878]
24. Bonham KS et al. (2014) A promiscuous lipid-binding protein diversifies the subcellular sites of toll-like receptor signal transduction. *Cell* 156(4):705–716 [PubMed: 24529375]
25. Holler N et al. (2003) Two adjacent trimeric Fas ligands are required for Fas signaling and formation of a death-inducing signaling complex. *Mol Cell Biol* 23(4):1428–1440 [PubMed: 12556501]
26. Micheau O, Tschoop J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* 114(2):181–190 [PubMed: 12887920]

Fig. 1.

Schematic layout of strategic planning for myddosome isolation from primary BMDMs and iBMDMs. Estimated time frame for myddosome isolation from primary BMDMs (left) and iBMDMs (right). Shared procedures are color coded. D stands for days

Fig. 2.

Myddosome formation induced by LPS in wild-type (WT) iBMDMs. Cells were stimulated with 1 $\mu\text{g/mL}$ LPS for indicated time points (30, 60, and 120 min), and were subsequently lysed. Myddosomes were isolated from cleared lysates by MyD88 immunoprecipitation. Components of the myddosome eluates (IRAK2, IRAK4, TIRAP, and MyD88) and input samples were separated by SDS- PAGE and were detected by western blotting. The membrane was developed with the Biorad ChemiDoc™ XRS+ System. Note: Asterisk (*) indicates the IgG light chain [IgG(L)]

Table 1

TLRs and their ligands

TLR	Microbial ligands ^a	Synthetic ligands
TLR1/TLR2	Triacylated lipopeptides	Pam3CSK4
TLR2	Bacterial Lipoproteins, Lipoarabinomannan, zymosan	
TLR3	dsRNA	poly(I:C)
TLR4	LPS	
TLR5	Flagellin	
TLR2/6	Diacylated lipopeptides; <i>Mycoplasma</i> MALP-2, FSL-1	Pam2CSK4
TLR7	ssRNA	Imiquimod, R848, Loxoribine
TLR8	ssRNA	R848
TLR9	Bacterial and viral CpG DNA, <i>Plasmodium</i> haemozoin	CpG ODNs
TLR10	ND	
TLR11	<i>Toxoplasma</i> Profilin; uropathogenic <i>E. coli</i> (UPEC)	
TLR12	<i>Toxoplasma</i> Profilin	
TLR13	Bacterial 23S ribosomal RNA	ORN Sa19

^aPlease note that this table focuses on representative ligands detected by TLRs, which does not represent an exhaustive list of TLR ligands