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Visualizing Toll-like Receptor-dependent Phagosomal Dynamics in Murine Dendritic Cells Using Live Cell Microscopy

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Abstract

Dendritic cells are professional phagocytes that are highly specialized to process and present antigens from internalized particles to prime naïve T cells. To achieve their functions, the phagocytic machinery and membrane dynamics of these cells have been adapted to optimize presentation of antigens from phagocytosed particles that bear ligands of pattern recognition receptors, such as toll-like receptors (TLRs), and that are thus perceived of as "dangerous". We have recently shown that phagosomes that are engaged in TLR signaling in dendritic cells emit numerous long tubules that facilitate content exchange with other signaling phagosomes and favor presentation of particle-derived antigens. This chapter describes the methods used to study the formation of these tubules, which we refer to as "phagotubules", by live cell imaging of mouse dendritic cells after the phagocytosis of fluorescent latex beads. We also describe methods to assess the effect of TLR signaling on this process.

Keywords

dendritic cells; phagocytosis; phagosome; phagotubules; toll-like receptors; live cell imaging; antigen presentation

1. INTRODUCTION

Dendritic cells (DCs) link innate immune signaling to adaptive immunity. Classical DCs (cDCs) are a subset of hematopoietic cells that reside both in lymphoid and non-lymphoid tissues, and are distinguished from plasmacytoid DCs that function largely as sources of type I interferon during infection (1–3). In peripheral tissues, cDCs continually sample the extracellular milieu for potential pathogens. Pathogens are sensed by a series of pattern recognition receptors (PRRs) that are present on the DC surface and/or within intracellular organelles (4–6). Triggering of these PRRs stimulates a maturation response that includes the release of proinflammatory and/or antiviral cytokines, migration to lymph nodes, and processing of internalized material for presentation to naïve T lymphocytes. This process

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provides the initial stimulus for adaptive immunity and tunes the immune response to the type of encountered pathogen (7, 8).

cDCs internalize pathogens and other extracellular material by different mechanisms. Soluble materials, including viruses and contents released from bacteria or parasites, are internalized by either receptor-mediated endocytosis or micro- or macropinocytosis, with the latter being highly up-regulated in immature cDCs (9, 10). cDCs are also among a number of professional phagocytic cell-types that are specialized to capture large particles such as bacteria by an actin-dependent mechanism known as phagocytosis (11). Upon completion of phagocytosis, the internalized particle is enclosed within a phagosome, an organelle that undergoes progressive remodeling through a series of interactions with the endosomal system (12). This remodeling process, referred to as phagosome maturation, provides a mechanism to kill invading particulate organisms and to break them down through hydrolysis. It also provides a means to allow signaling via phagosome-associated PRRs, including Toll-like receptors (TLRs), following recruitment of PRRs and their signaling platforms (8)

Phagosome maturation manifests distinct features in different phagocytic cell types. In cDCs, the maturation of both phagosomes and classical endosomes is skewed toward preservation of antigenic peptides for assembly with major histocompatibility complex (MHC) class I (MHC-I) and class II (MHC-II) molecules for presentation to T cells. For example, maturing phagosomes and endolysosomes in cDCs are less acidic than in other cell types, at least in part due to impaired assembly of the vacuolar ATPase and to the consumption of protons by superoxide generated by a highly active NADPH oxidase (13, 14). This restrains proteolysis by lysosomal hydrolases and thus prolongs survival of antigenic peptides. In addition, cDCs are specialized to form tubules from both endolysosomes and phagosomes following TLR stimulation. Long tubules that emerge from endolysosomes following stimulation with soluble TLR ligands favor the delivery of newly assembled MHC-II:peptide complexes to the DC plasma membrane and towards the immunological synapse with T cells (15, 16). Similarly, we have shown that tubules form from maturing phagosomes in cDCs several hours after uptake of bacteria or TLR ligand-coated latex beads (17). In contrast to the endolysosomal tubules, these “phagotubules” are not essential for the transport of MHC-II:peptide complexes to the cell surface and do not appear to fuse with the plasma membrane. Rather, they favor content exchange among distinct TLR signaling phagosomes within the same DC. Content exchange favors optimal antigen presentation, likely by exposing MHC-II molecules on multiple phagosomes to more antigen sources (17).

TLRs such as TLR2 and TLR4 can signal from phagosomes independently of plasma membrane TLRs (18–20). They trigger distinct signaling pathways through two sets of adaptors: TIRAP-MyD88 and TRAM-TRIF (21). Upon TLR stimulation at the plasma membrane or phagosomes, MyD88 recruits IL-1 receptor-associated kinases (IRAK) 1 and 4. Subsequent activation of IRAK4 and IRAK1 initiates a signaling cascade that ultimately activates MAP kinases and the transcription factors AP-1 and NF-κB. TLR activation of the TRAM-TRIF pathway on endosomes or phagosomes specifically induces the phosphorylation of the transcription factor interferon regulatory factor-3 (IRF-3) by

activating the protein kinases TANK-binding kinase-1 (TBK-1) and IKK-I (22–24). The importance of each of these pathways from phagosomes or other cellular sites for a variety of downstream responses can be dissected by exploiting mice that lack functional genes encoding the adaptors MyD88 (25), TIRAP/MAL (26), TRIF (27) and TRAM (28) or by using pharmacological inhibitors of IRAK1/4 or TBK-1. Because of the inducible formation of phagosomes following particle uptake, addition of pharmacological inhibitors at different times following exposure to TLR ligand-coated particles can be used to distinguish signaling from the plasma membrane and from the phagosomes themselves.

In the following sections we will describe the procedure that we have used to visualize the formation of tubules from phagosomes by live cell imaging of dendritic cells and to define the role of downstream effectors of TLR4 signaling on this process. We will focus on bone marrow-derived DCs (BMDCs) as a model system. These cells represent a population of $CD11b^+/CD11c^+/B220^-$ cells that resemble *in vivo* inflammatory or migratory DCs (1, 29). This procedure can also be applied to resident DCs purified from different tissues such as mouse spleen.

2. MATERIALS

2.1. Bead coating and phagocytosis

1. BMDC complete medium: RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, Defined FBS, cat. # SH30070.03), 2 mM glutamine, 100 U/ml penicillin/ 100 μg/ml streptomycin and 50μM 2-mercaptoethanol. For BMDC culture conditions see Note 1.
2. BMDC conditioned-complete medium: Same as BMDC complete medium with 30% granulocyte-macrophage colony stimulating factor (GM-CSF)-transfected NIH-3T3 fibroblast conditioned medium containing 10–20 ng/ml GM-CSF (Winzler, C. et al. J Exp Med 185, 317–28, 1997); See Note 2). Alternatively, recombinant granulocyte-macrophage colony stimulating factor can be provided directly at 1000 U/ml (R&D Systems, Minneapolis, MN).
3. Phosphate buffered saline without $CaCl_2$ or $MgCl_2$ (PBS).
4. 0.25% Trypsin-EDTA (Life Technologies, cat # 25200-052).
5. Poly-L-Lys coated glass bottom 35 mm culture dishes (MatTek, Ashland, MA, cat. # P35G-1.5-14-C).

[1]BMDCs were cultured following the procedure described by Winzler et al. (31) for D1 and splenic DCs. Briefly, cells were flushed from mice tibias and femurs and seeded on 10 cm non-tissue culture-treated Petri dishes at a concentration of 1×10^6 cells/ml and passaged twice a week using trypsinization for 7–10 days. Alternatively, BMDCs can be cultured according to Inaba et al. (32). For a detailed protocol on bone marrow cell isolation and BMDC culture see Gross (33). The characteristic phenotype of BMDCs should be analyzed by flow cytometry as described by Winzler et al. (31). The cell population should be between 80–90% $CD11b^+/CD11c^+$ (BMDCs) at the time of the experiment. In an immature state, BMDCs display low staining for major histocompatibility complex class II molecules (MHC-II) and the costimulatory molecules CD86 and CD80. Maturation is initiated by the addition of LPS (100 ng/ml) or LPS-coated beads; 16–24 h. later, MHC-II and costimulatory molecule expression strongly increases.

[2]Aliquot and store GM-CSF-containing supernatant at −80°C for multiple uses. Once thawed, the supernatant can be stored at 4°C for approximately one month.

6. Polybead polystyrene 3 μm microspheres (Polysciences Inc,, Warrington, PA, cat. # 17134).
7. Polybead amino 3 μm microspheres (Polysciences Inc., Warrington, PA, cat. # 17145).
8. Lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 strain (Sigma, St. Louis, MO, cat. # L4391 or Invivogen, San Diego, CA, cat. # tlrl-3pelps). Prepare as 1 mg/ml stock solution in endotoxin-free phosphate-buffered saline (PBS, Life Technologies, cat. # 14190-136) or water (Life Technologies, cat. # 15230-147). The stock can be stored at 4°C and diluted to 100 μg/ml for the working solution.
9. Polyinosine-polycytidylic acid (poly(I:C)) (Invivogen, cat. # tlrl-pic). Prepare as 1 mg/ml stock solution in endotoxin-free water and dilute to 100 μg/ml for the working solution. Aliquot and store stock solution at −20°C.
10. Ovalbumin (OVA) conjugates (Life Technologies-Molecular Probes; Texas Red ovalbumin, cat. # O-23021; Alexa Fluor 488 ovalbumin, cat. # O-34781). Prepare as 5 mg/ml stock solution in endotoxin-free PBS; use as 1 mg/ml working solution. Aliquot and store stock solutions at −20°C.
11. 8% Glutaraldehyde (Polysciences).
12. 0.5 M Glycine in PBS.

2.2. Pharmacological treatments

1. Interleukin-1 Receptor-Associated Kinase-1/4 (IRAK-1/4) inhibitor (EMD Biosciences, Billerica, MA, cat. #407601). Prepare 12.6 mM stock in DMSO; use as 5–50 μM working solution in BMDC complete medium. Aliquot and store stock solution at −20°C.
2. TBK1/IKKε inhibitor BX795 (Invivogen, cat. #tlrl-bx7). Prepare 10 mM stock solution in DMSO; use as 0.1 μM working solution in BMDC complete medium. Aliquot and store stock solution at −20°C.
3. 3-Methyladenine (3-MA) (Sigma, cat. # M9281). Prepare 100 mM stock solution in PBS with heating the day of the experiment; use as 5 mM working solution in BMDC complete medium.
4. Bafilomycin A1 from *Streptomyces griseus* (Sigma, cat. # B1793). Prepare 100 μM stock solution in DMSO; use as 0.5 μM working solution in BMDC complete medium. Aliquot and store stock solution at −20°C.
5. Methyl-(5-[2-thienylcarbonyl]-1 H-benzimidazol-2-YL) carbamate (nocodazole, Sigma, cat. # M1404). Prepare 16.6 mM stock solution in DMSO; use as 10–20 μM working solution in BMDC complete medium. Aliquot and store stock solution at −20°C.
6. Latrunculin B from *Latruncula magnifica*. (Sigma, cat. # L5288). Prepare 1 mM stock solution in ethanol; use as 1 μM working solution in BMDC complete medium. Aliquot and store stock solution at −20°C.

7. Cytochalasin D (Sigma, cat. # C8273). Prepare 10 mM stock solution in DMSO; use as 1 μM working solution in BMDC complete medium. Aliquot and store stock solution at −20°C.

2.3. Detection of phosphorylated proteins

1. Antibody to interferon regulatory factor (IRF)-3 (Cell Signaling, Beverly, MA, cat. # D83B9).
2. Antibody to phospho-interferon regulatory factor (IRF)-3 (Ser396) (Cell Signaling, 4D4G rabbit monoclonal antibody).
3. Antibody to p38 MAP kinase (Cell Signaling, cat. #**9212**)
4. Antibody to phospho-p38 MAP kinase (Cell Signaling, cat. #**9211**)
5. Either phosphatase inhibitor cocktail tablets (PhosStop, Roche Diagnostics, Indianapolis, IN) or solution containing 2 mM Na_3VO_4, 25 mM NaF, 5mM β-glycerophosphate.
6. Ice cold PBS without $CaCl_2$ or $MgCl_2$.
7. 96 well round bottom plates.

3. METHODS

3.1. Coating of OVA fluorophore-conjugated to 3 μm amino beads

This procedure has been adapted from (30).

1. Calculate the amount of beads required for the experiment. Plan to use 6 μl of bead suspension per condition to test, per time point. Consider performing each condition in triplicates or more to allow for imaging at different time points. You may need to perform preliminary experiments to test the amount of beads needed such that the average cell will take up the desired number of beads. Phagotubules are best observed when cells take up 4–6 beads per cell.
2. Resuspend bead suspension in 1 ml PBS per 100 μl of suspension.
3. Centrifuge for 5 min. in a microcentrifuge at 4°C at maximum speed (14,000 rpm/ 20,000 x g in an Eppendorf 5417R microcentrifuge). Remove supernatant carefully and discard it.
4. Add 1 ml PBS and centrifuge as in 3.
5. Repeat Step 4 once more.
6. Activate the beads for conjugation by resuspending them in 0.5 ml of 8% glutaraldehyde.
7. Mix for 4–6 h at room temperature on an end-to-end mixer (preferably a rotating wheel, but a shaker will suffice).
8. Centrifuge 5 min at 14,000 rpm and discard supernatant.
9. Repeat Step 4 once.

10. Add 1 mg/ml of fluorescent OVA conjugate and 100 μg/ml LPS in 0.5 ml PBS final volume.
11. Mix overnight at 4°C with end-to-end mixing (a rotating wheel is preferred).
12. Centrifuge 5 min. at 14,000 rpm and discard supernatant.
13. Resuspend pellet in 1 ml of 0.5 M glycine and mix for 30 min at room temperature. This step is performed to block unreacted sites on the beads.
14. Centrifuge 5 min at 14,000 rpm and discard supernatant.
15. Repeat Step 4 twice. Fluorescent OVA is now covalently conjugated to the beads.
16. Resuspend the pellet in the original volume of beads. Fluorescent OVA-coated beads can be stored for 1 week at 4°C.

3.2. Phagocytosis of OVA fluorophore conjugated- coated latex beads

1. Differentiate and culture BMDCs in BMDC conditioned-complete medium for 7–10 days on non tissue culture treated plastic dishes, as previously described (see Note 1). For isolation of mouse tissue-resident DCs see Note 3.
2. On day 6 of BMDC culture, harvest BMDCs by washing the dishes once with PBS and then treating them with trypsin-EDTA for no longer than 5 min (see Note 4). Collect detached cells and recover by centrifugation for 5 min at 150 x g (1,000 rpm in a Sorvall ST40R table top centrifuge with a TX-750 rotor). Resuspend the cells at 200,000 BMDCs/ 2 ml of BMDC conditioned-complete medium for each sample to be analyzed. Seed 2 ml/ dish in poly-L-Lys-coated 35 mm culture dishes. Seed 1 dish per condition per time point, to allow for imaging at different time points.
3. On day 7, remove medium and add 1.5 ml of BMDC complete medium without or with inhibitors. Add inhibitors either 2.5 h before the pulse, at the time of the pulse or at the start of the chase at the concentrations indicated above (see Note 5 regarding testing the efficacy and specificity of the inhibitors). Use DMSO at a comparable dilution as vehicle control.
4. Pulse the cells with beads. Carefully remove media from the dishes and add 1.5 ml of BMDC complete medium (plus/ minus inhibitors) plus OVA-Texas Red and/or OVA-Alexa Fluor 488-coated beads at a 1:200 dilution.
5. Leave dishes on ice for 15–30 min to allow the beads to adhere to the cells.
6. Incubate at 37°C for 30 min to allow for phagocytosis.

[3]Tissue resident DCs can be isolated by tissue homogenization followed by purification with Miltenyi antibody-coated beads following the manufacturer instructions (Miltenyi Biotec, San Diego, CA). Cells are then plated and treated as described for BMDCs.
[4]BMDCs can be harvested using trypsin-EDTA or with PBS without $CaCl_2$ or $MgCl_2$ alone. Trypsin-EDTA is preferred when passaging cells in culture and when cells will not be used for experiments within 24 h after trypsinization. After a 5 minute treatment with trypsin-EDTA, cells that remain attached are considered macrophages and discarded. See Note 5.2 for BMDC harvesting with PBS for experiments performed on the same day of the cell harvest.

trigger both the TIRAP-MyD88 pathway and the TRAM-TRIF pathway. To test for TBK-1 inhibitor efficacy specifically, use a TLR3 ligand (like poly(I:C)-coated beads) that stimulates only the TRAM-TRIF pathway. An alternative to measuring cytokine secretion is to monitor phosphorylation of downstream effectors, such as IRF3 after stimulation of the TRAM-TRIF axis or p38 MAP Kinase after stimulation of the TIRAP-MyD88 axis. Below we describe these procedures:

5.1 Coating of LPS or poly(I:C) to polystyrene beads

1. Calculate the amount of coated beads required for the experiment. Plan to use 3 μl of bead suspension per condition, in triplicates, per time point. For assaying by ELISA, consider testing at least 2 time points. For assaying by immunoblotting, consider testing at least 3 time points. Leave some beads uncoated, washed 3 times with PBS (steps 2–5 below), to be used as a negative control.
2. For coating: resuspend bead suspension in 1 ml PBS per 100 μl of suspension.
3. Centrifuge for 5 min in a microcentrifuge at 14,000 rpm, 4°C. Remove supernatant carefully and discard it.
4. Add 1 ml PBS and centrifuge as in 3.
5. Repeat Step 4 once more.
6. Add LPS or poly(I:C) to a final concentration of 100 μg/ml in 1 ml of PBS.
7. Mix overnight at 4°C with end-to-end mixing (a rotating wheel is preferred).
8. Centrifuge at 14,000 rpm for 5 min at 4°C in the microcentrifuge and discard supernatant.
9. Repeat Step 4 3 times.
10. Resuspend the pellet in the original volume of beads. The coated beads are now ready to use.

5.2 Detection of IL-6 and IL-12 secretion by ELISA

1. On days 7–10 of BMDC culture, collect BMDCs using PBS only and without trypsinization (see Note 4) as follows. Transfer the culture medium containing BMDCs in suspension to a 50 ml conical tube. Collect mildly adherent BMDCs by adding 5 ml of ice cold PBS to each 10 cm dish and rigorous pipetting, and transfer the resuspended cells in PBS into the same 50 ml tube. Finally, add 8 ml of ice cold PBS to each dish and incubate for 30 min at 4°C. Rigorously pipette again and transfer the final suspension into the same conical tube.
2. Centrifuge 5 min, 1,000 rpm, at 4°C.
3. Discard supernatant and resuspend BMDCs in BMDC complete medium. Count BMDCs.
4. Seed BMDCs into 96 well round-bottom plates (1 per time point) at 60,000 BMDCs/ well in 50 μl of BDC complete medium. Plate enough wells to test the following conditions in triplicate: (1) unstimulated (no beads added); (2) stimulated with uncoated beads; (3) stimulated with LPS coated beads; and (4) stimulated with poly(I:C) coated beads. Inhibitors or vehicle control should be tested under all stimulating conditions.
5. Add 50 μl of BMDC complete medium to the untreated or “after pulse” conditions, and add inhibitors as 2X concentrates in 50 μl of BMDC complete medium for all treated samples in which agents are to be added 2 h before or at the pulse.
6. Dilute bead suspension 1:16.6 in BMDC complete medium (3X bead suspension). Separate the bead suspension volumes required for the conditions with inhibitors, and add the corresponding inhibitors (1X concentrated).
7. Add 50 μl of 3X bead suspension to each well (final dilution 1:50).
8. After 30 min. add 50 μl of 4X concentrated inhibitors in BMDC complete medium to the “after pulse” condition. Add 50 μl of 1X concentrated inhibitors in BMDC complete medium to the “before pulse” and “at pulse conditions” and 50 μl of BMDC complete medium to the untreated conditions. Final volume: 200 μl.
9. Incubate plates at 37°C for 3 and 6h.
10. Collect supernatants and transfer them to 96 well round bottom plates. Store them at −20°C until use.
11. Perform ELISA according to manufacturer’s instructions. Dilute supernatants 1/2, 1/4 and 1/8.

5.3 Detection of phosphorylated proteins by Western blot

1. Collect BMDCs with PBS, as described above.
2. Seed cells on 96 well round-bottom plates (1 per time point) at 100,000 BMDCs/well in 50 μl of BMDC complete medium. Conditions to test (in triplicates): unstimulated, uncoated bead-stimulated, LPS coated bead-stimulated, poly(I:C) coated bead-stimulated; all conditions treated with the inhibitors to test, or left untreated.
3. Perform steps 5–8.
4. Incubate plates at 37°C for 1h, 1.5 h and 3h.
5. #x02022; 5. Remove supernatants. They can be stored at −20°C to test for cytokine secretion.
6. Add cold PBS to plates, and centrifuge 5 min. at 150 x g.
7. Discard supernatants and add 30 μl of Laemmli sample buffer 1X containing phosphatase inhibitors to the wells.
8. Store plates at −20°C until use.
9. Analyze samples by 10% SDS-PAGE and Western blot. Run 2 separate gels, one to detect IRF3 and the other one for P-IRF3.

7. Carefully remove media and wash dishes 3 times with RPMI 1640 medium.

(without FBS) to remove non-phagocytosed beads. Add BMDC complete medium for the 2 h chase.

8. Analyze by live cell imaging using an inverted fluorescence microscope (optimally a spinning disk confocal microscope) equipped with an environmental chamber kept at 37°C and 5% CO_2. If addition of CO_2 is not possible, add 20 mM HEPES buffer (pH 7.4) to the cells immediately before imaging. See Note 6 regarding imaging requirements. Figure 1 shows examples of single frames from movies taken at 1 frame/second using an Olympus IX71 inverted spinning disk confocal microscope equipped with a Hamamatsu ImagEM EMCCD camera and LCI Chamlide stagetop incubation system for live cell imaging. Movies were acquired over a 5 min time period using MetaMorph software (Molecular Devices, LLC, Sunnyvale, CA, USA). The image sequences were saved as TIF files, and then further analyzed using Image J (NIH) as described below.

3.3. Quantification of tubule number and length using Image J software

See Note 7 for alternative analysis options.

1. Open the image sequence file in .tiff extension. Quick time movie format can be opened in the 32-bit version of Image J.
2. Go to “Image”, “Type” and set to 8-bit.
3. Go to “Process”, “Binary” and click on “Make binary”.
4. Go to “Process”, “Binary” and click on “Skeletonize”. This mode allows for a better visualization of tubular structures by stylizing the images. If the tubules are predominantly arranged independently of each other, they can be analyzed automatically as described below in step 5. If the tubules are interconnected in a network structure, they must be analyzed manually. This is described in step 6 below.
5. Go to “Analyze” and click on “Analyze particles” to count tubules of the desired length on each movie frame (for example, you might want to count tubules that are greater or shorter than 1 μm in length). A dialog box will appear. Type the tubule length and the program will count structures of the indicated length on each movie frame. When the program completes the command, it will show a “Summary” of

[6]Due to the fast-changing dynamics of tubule formation, extension and contact with neighboring phagosomes, the use of spinning disk confocal microscopy is advised to allow for rapid acquisition of images in a single plane (at least 1 frame/second). This is particularly important under circumstances in which multiple fluorescence channels are analyzed and/or in which bright field, phase contrast, or differential interference contrast (DIC) detection is used to better visualize cell morphology in addition to the fluorescence associated with the beads. Environmental conditions that ensure cell health are also required, as cellular stresses impact poorly on membrane dynamics. Therefore, a microscope equipped with an environmental chamber providing stable temperature and 5% CO_2 is strongly suggested. See details above for specifications on the microscope that we have used in our studies.

[7]Multiple software packages are available for image acquisition and analysis, and most would be suitable for acquiring and analyzing the formation of tubules from phagosomes or endolysosomes. Image J is a versatile program that is supported by the National Institutes of Health and can be downloaded for free from the NIH website (http://imagej.nih.gov/ij/); FIJI (FIJI Is Just ImageJ) is a complete Image J package with multiple useful Plugins and a convenient interface, and is available for free download at http://fiji.sc/Fiji. Another useful tool for analysis is Matlab software (MathWorks, Natick, MA), which allows for the development and application of algorithms to track particles and detect particle merging and splitting events in live-cell time-lapse sequences. A useful tracking software for Matlab platform is u-track 2.0, which is available online from Harvard Medical School (http://lccb.hms.harvard.edu/software.html).

the number of tubules of that length per frame. If any tubules are interconnected within a network, the network will be counted as a single tubule.

6. Because a network of interconnected tubules are counted as a single tubule using “Analyze particles”, movie frames with interconnected tubules are best counted manually in each frame. To do this, advance the movie frame by frame. Go to “Plugins”, “Cell counter”. A window will let you select among different counter symbols; hence you can select one counter symbol per tubule length that you wish to count (for example, tubules that are greater or shorter than 1 μm in length). Click on every tubule on each frame. Then go to “Results” to visualize a chart with a list of tubule counts of each length category per frame.

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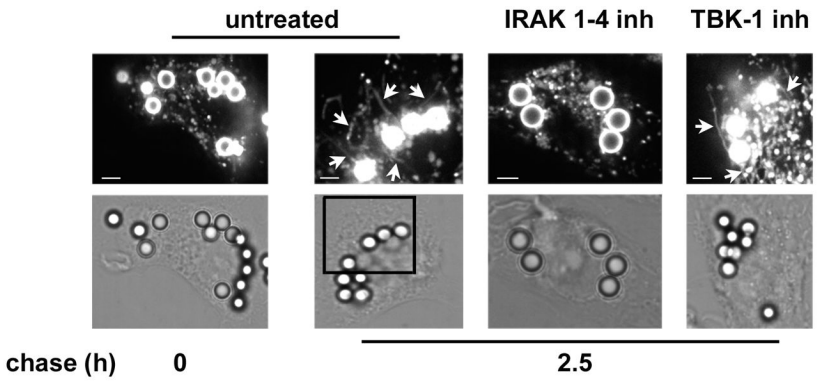

Fig. 1.

Visualizing BMDC phagotubules and the effects of inhibitors of TLR signaling on their formation. BMDCs from C57BL/6 mice were left untreated or were pre-treated for 2 h with 5 μM IRAK 1-4 inhibitor (inh) or 0.1 μM BX795 (TBK-1 inh). Cells were then pulsed for 15 min with OVA-Texas Red coated beads and chased for 2.5 h, all in the absence or presence of inhibitor as indicated. Cells were then imaged by spinning disk confocal microscopy. Shown are still images of Texas Red fluorescence (top) and corresponding DIC images (bottom). Bars, 2.6 μm.