Video Article

Detection of Neu1 Sialidase Activity in Regulating TOLL-like Receptor Activation

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Abstract

Mammalian Toll-like receptors (TLRs) are a family of receptors that recognize pathogen-associated molecular patterns. Not only are TLRs crucial sensors of microbial (e.g., viruses, bacteria and parasite) infections, they also play an important role in the pathophysiology of infectious diseases, inflammatory diseases, and possibly in autoimmune diseases. Thus, the intensity and duration of TLR responses against infectious diseases must be tightly controlled. It follows that understanding the structural integrity of sensor receptors, their ligand interactions and signaling components is essential for subsequent immunological protection. It would also provide important opportunities for disease modification through sensor manipulation. Although the signaling pathways of TLR sensors are well characterized, the parameters controlling interactions between the sensors and their ligands still remain poorly defined. We have recently identified a novel mechanism of TLR activation by its natural ligand, which has not been previously observed [1,2]. It suggests that ligand-induced TLR activation is tightly controlled by Neu1 sialidase activation. We have also reported that Neu1 tightly regulates neurotrophin receptors like TrkA and TrkB [3], which involve Neu1 and matrix metalloproteinase-9 (MMP-9) cross-talk in complex with the receptors [4]. The sialidase assay has been initially use to find a novel ligand, thymoquinone, in the activation of Neu4 sialidase on the cell surface of macrophages, dendritic cells and fibroblast cells via GPCR Gαi proteins and MMP-9 [5]. For TLR receptors, our data indicate that Neu1 sialidase is already in complex with TLR-2, -3 and -4 receptors, and is induced upon ligand binding to either receptor. Activated Neu1 sialidase hydrolyzes sialyl α-2,3-linked β-galactosyl residues distant from ligand binding to remove steric hinderance to TLR-4 dimerization, MyD88/TLR4 complex recruitment, NFkB activation and pro-inflammatory cell responses. In a collaborative report, Neu1 sialidase has been shown to regulate phagocytosis in macrophage cells [6]. Taken together, the sialidase assay has provided us with powerful insights to the molecular mechanisms of ligand-induced receptor activation. Although the precise relationship between Neu1 sialidase and the activation of TLR, Trk receptors has yet to be fully elucidated, it would represent a new or pioneering approach to cell regulation pathways.

Video Link

The video component of this article can be found at https://www.jove.com/video/2142/

Protocol

1. Resurrecting Frozen Macrophage Cells

1. Before resurrecting frozen cells from the -80°C freezer, one needs to prepare culture medium using sterile filtered Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 5 µg/mL of plasmocin. Plasmocin is an antibiotic solution used in our research to eliminate and prevent mycoplasma contamination of cell cultures.
2. For one vial of frozen cells, one adds 4 mL, 1 mL of the cultured medium to a 25 cm^2 cell culture flask in a sterile biohazard containment hood.
3. When the vial of frozen cells are taken from the -80°C freezer, they are quickly thawed by hand warming in the sterile hood, which takes about 2-3 min.
4. As soon as the cells are thawed, they are quickly transferred into the flask containing the conditioned medium using a 1mL sterile pipette.
5. The flask containing the cells are placed in an tissue culture incubator at 37°C and 5% CO_2.
6. The cells checked for growth and adherence on a daily basis until they become 75% to 90% confluent using an inverted microscope.
7. During the cell culture incubation, 12 mm circular glass slides are sterilized by covering them in 70% ethanol in a large glass petri dish for 1 hr in the biohazard containment cabinet, the alcohol is afterwards removed and stored for reusage, and the wet glass sides are left exposed to the sterile-filtered air flow in the cabinet under a UV for 24 hrs or until they are dry.

2. Plating Cells for the Sialidase Assay

1. Before plating the cells, one single sterile circular glass slide is carefully placed in one well of a BD Falcon 24-well flat-bottom with lid tissue-culture treated polystyrene plate using a sterilized flaming full curved, 4", serrated, stainless steel forcep. For one flask of cells at 75% confluence, we usually use 12 wells of the culture plate.
2. For adherent macrophage cells, the DMEM conditioned medium is removed from the flask using a 5 mL sterile pipette in the sterile containment cabinet. Wash the adherent cells once with 1x sterile Tris buffered saline pH 7.4 to remove any serum containing medium, and add 1 mL of calcium-medium free (CMF) phosphate buffered saline at pH 7.4 enough to cover the cells. The cells are placed in the 37°C incubator for approximately 10 min or until the cells start to lift off in the flask. The flask can be gently rocked or shaken to loosen any remaining adherent cells.
3. To the 1 mL of suspended cells in the flask, add 3 mL of DMEM conditioned medium. Take 0.5 mL of the suspended cells and sterilely transferred them to the tissue cultured plate containing the 12 mm circular glass slides. Approximately, 0.5 x 10^6 cells are transferred to the circular glass slides.
4. The tissue culture plate containing cells are incubated at 37°C in 5% CO_2 for 24 hrs in order to allow for the cells to adhere to the circular glass slides.

3. Sialidase Assay

1. Making the Control

1. Place a microscope slide (frosted: 1 end, 1slide, 25 x 75 x 1 mm) in the containment cabinet or lab bench and add by mixing in sequential order the following compounds: 1 µL DAKO + 1 µL of 4-MUNANA and lastly + 3 µL of Tris buffered saline pH 7.4 (TBS).
2. After removing the media from one well containing the cells, gently lift the circular glass slide with the 4" forcep to the corner of the well. Carefully remove the glass slide with the forcep and place the slide with the cells facing onto the mixture solution on the microscope slide.
3. Immediately, take the microcope slide to an epi-fluorescent microscopy which has been set for the UV filter and has a digital camera for capturing the fluorescent images.
4. Focus the microscope under phase contrast until you can see the cells and then switch to fluorescent mode. Take pictures of the cells under fluorescent at 1 min, 2 min, and 3 min. Take a single picture of the cells under phase contrast.

2. Making the Positive Test

1. Spot the following compounds in the sequential order on another microscope slide: 1 µL DAKO + 1 µL of 4-MUNANA + 2 µL of LPS + 1 µL of TBS.
2. Repeat steps 3.1.2 to 3.1.4 above.

3. Making the Positive Test together with Neuraminidase inhibitor Tamiflu

1. Spot the following compounds in the following sequential order on another microscope slide: 1µL DAKO + 1µL of 4-MUNANA + 2 µL of LPS + 1 µL of Tamiflu.
2. Tamiflu concentrations are pre-prepared in 1x Tris buffered saline pH 7.4; the final concentration of the compounds in contact with the live cells on the circular glass slide will have a dilution factor of 5.
3. Repeat steps 3.1.2 to 3.1.4 above.

4. Determination of the Concentration of Inhibitor needed to Inhibit 50% of the Sialidase Activity (IC_{50})

1. To quantify the fluorescent surrounding the cells, the images are analyzed using ImageJ 1.38x with the analyze plugin for measuring RBG. Take 50 random spot readings of the fluorescence surrounding the cells and calculate the mean of the total fluorescence.
2. The IC_{50} is generated from a plot of the inhibitor concentration converted as log units (e.g., log ng/mL) on the x-axis against the mean fluorescence on the y-axis using nonlinear regression.

5. Secrets to Success

1. Make sure the cells in culture are contamination free from mycoplasma. We routinely use Plasmocin in a culture medium to control for this.
2. The artificial neuraminidase substrate, 4-MUNANA, should be used freshly prepared. The prepared substrate can be used within a week for the sialidase assay.
3. If the cell line has a low receptor expression on the cell surface, one can use a higher dose of the ligand for stimulation.
4. We have also experienced that cells passaged for over 6 times might lose their receptor phenotype. Freshly, resurrected cells should be cultured.

6. Representative Results

See animated protocol of the sialidase assay with representative results in the attached Powerpoint file.

Discussion

Using the newly developed assay to detect sialidase activity in live macrophage cells [2], we used this technology to detect sialidase activity in ligand-induced sialidase activity in live BMC-2 macrophage cells in a dose dependent manner as well in live DC-2.4 dendritic cells, HEK-TLR4/MD2, HEK293, SP1 mammary adenocarcinoma cells, human WT and 1140F01 and WG0544 type I sialidosis fibroblast cells. Neuraminidase inhibitors like Tamiflu (oseltamivir phosphate) inhibited thymoquinone-induced sialidase activity in live BMC-2 cells with an IC_{50} of 0.0194 µM compared to an IC_{50} of 19.17 µM for neuraminidase inhibitor DANA (2-deoxy-2,3-dehydro-D-N-acetylneuraminic acid) [5]. We have also reported that other applications such as specific anti-Neu1, -2 and 3 antibodies have no inhibition of TQ-induced sialidase activity in live BMC-2 and human THP-1 macrophage cells but anti-Neu4 antibodies completely block this activity. There is an application of the sialidase activity to detect a vigorous sialidase activity associated with TQ treated live primary bone marrow (BM) macrophage cells derived from WT and hypomorphic cathepsin A mice with a secondary Neu1 deficiency (Neu1 KD) but not from Neu4 knockout (Neu4 KO) mice [1,2,5]. In addition, pertussis toxin (PTX), a specific inhibitor of Gαi proteins of G-protein coupled receptor (GPCR) and the broad range inhibitors of matrix metalloproteinase (MMP) galardin and piperazine applied to live BMC-2, THP-1 and primary BM macrophage cells completely block TQ-induced sialidase activity [5]. These same inhibitory effects are not observed with the GM1 ganglioside specific cholera toxin subunit B (CTXB) as well as with CTX, tyrosine kinase inhibitor K252a, and the broad range GPCR inhibitor suramin. The specific inhibitor of MMP-9, anti-MMP-9 antibody and anti-Neu4 antibody but not the specific inhibitor of MMP-3 completely block TQ-induced sialidase activity in live THP-1 cells which express Neu4 and MMP-9 on the cell surface [5].

Taken together, the sialidase assay can be used to provide powerful insights to the molecular mechanisms of ligand-induced receptor activation involving sialidases like Neu1 or Neu4 depending on the nature of the ligand. The rapidity of the ligand-induced sialidase activity mediated by the ligand-bound receptor suggests that glycosylated receptors like NGF TrkA, BDNF TrkB and TOLL-like receptors form a signaling paradigm on the cell surface membrane involving a molecular organizational platform of ligand-bound receptor, Gαi proteins, MMP-9 and Neu1 or Neu4 sialidase. Ligand binding respective receptors induces sialidase activity through GPCR-signaling via membrane Gαi proteins and MMP-9 activation. Neu1 or Neu4 and MMP-9 cross-talk in complex with the receptor on the cell surface enables a rapid activation of the sialidase to remove sialic acid-steric hinderance to receptor association in generating a functional receptor.

Disclosures

S.R.A. and P.J. contributed equally as first authors.

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