

Chapter 21

Allergens and Activation of the Toll-Like Receptor Response

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Abstract

Pattern recognition receptors (PRRs) provide a crucial function in the detection of exogenous and endogenous danger signals. The Toll-like receptors (TLRs) were the first family of PRRs to be discovered and have been extensively studied since. Whilst TLRs remain the best characterized family of PRRs there is still much to be learnt about their mode of activation and the mechanisms of signal transduction they employ. Much of our understanding of these processes has been gathered through the use of cell based signaling assays utilizing specific gene-reporters or cytokine secretion based readouts. More recently it has become apparent that the repertoire of ligands recognized by these receptors may be wider than originally assumed and that their activation may be sensitized, or at least modulated by the presence of common household allergens such as the cat dander protein Fel d 1, or the house dust mite allergen Der p 2. In this chapter we provide an overview of the cell culture and stimulation processes required to study TLR signaling in HEK293 based assays and in bone marrow-derived macrophages.

Key words TLR, Allergy, Fel d 1, Luciferase assay, Bone marrow-derived macrophage, Innate immunity, Signal transduction, NFκB, HEK293

1 Introduction

Knowledge of how TLRs are activated and how they propagate signaling is crucial for understanding the innate immune system, for the development of novel therapeutics against inflammatory diseases, and for the creation of new vaccine adjuvants [1]. The ability to manipulate and mutate the receptors, and their adaptor proteins, in order to probe molecular function at the level of individual amino acids has been highly informative and continues to be a mainstay in studies of receptor function. Interpretation of the results from these studies must always be done in full consideration of the caveat that these are overexpression systems making use of transient transfection of mutant receptors into potentially non-physiological cell types. It is therefore important, wherever possible, to validate observations using more physiological cell types

such as macrophages. This is particularly relevant when looking at ligand-mediated activation as the doses required to activate the two systems are often disparate.

In addition to the classical pathogen-derived TLR ligands it has recently become apparent that allergens also interface with TLRs to modulate their signaling [2]. These may be proteinaceous ligands such as the dust mite allergen Der p 2 [3, 4] or the cat dander protein Fel d 1 [5]; or they may constitute chemical allergens such as the metal nickel [6, 7]. The connections of these allergens with disabling conditions such as asthma [8, 9] and contact hypersensitivity has led to an upsurge in research aimed at understanding how they interface with TLRs and how they contribute to the initiation of TLR signaling. In order to understand the connection between allergens and TLR signaling researchers are employing both gene reporter based assays and the stimulation of primary cells such as bone marrow-derived macrophages from mice or primary human antigen presenting cells.

The use of gene reporters is widespread in biology, particularly in studies investigating changes in gene expression, promoter function, signaling pathways, activation or inhibition of receptor signaling, and the effect of transcription factors. Reporter assays have retained their status as a key experimental tool in these systems despite the increased accessibility of array based technology, quantitative reverse transcriptase PCR, and next generation RNA sequencing. This reflects not only the inherent robustness and flexibility of the assays, but also the fact that they don't require complex or highly expensive pieces of equipment. Most reporter assays utilize a dual reporter system in which changes in the expression of two reporter enzymes (such as luciferase or secreted embryonic alkaline phosphatase) are measured. One enzyme is placed under the control of a promoter that will respond to changes in the system of study—this is often nuclear factor kappa B or interferon response factors for TLR signaling pathways. This is the experimental reporter. The other enzyme is placed under the control of a promoter that will be expressed at basal levels in the cell population and serves as an internal control for variation in transfection efficiency and cell death. This is the control promoter. Normalizing the activity measured for the experimental reporter by that measured for the control reporter allows a much more robust and reliable interpretation of the experimental data.

One of the most common dual reporter systems used in cell signaling studies is the Dual-Luciferase Reporter (DLR™) Assay System developed by Promega [10]. This system utilizes two separate luciferases, one from the firefly (*Photinus pyralis*) and the other from the sea pansy *Renilla* (*Renilla reniformis*). The firefly gene, under the control of a suitable promoter sequence, serves as the experimental reporter and the *Renilla* luciferase, controlled by a constitutively active promoter, acts as the internal control reporter. The activity of both luciferases is measured sequentially, but rap-

idly, as a stabilized signal from the same sample, with the firefly luciferase being measured first. The assay is linear in nature and extremely sensitive with detection thresholds reported to be at the subattomole level [10]. A major advantage of these reporters is the lack of any endogenous signal.

It is often difficult, or time-consuming, to develop reporter systems in primary cells. Consequently, receptor signaling in these systems is often measured through the detection of secreted cytokines such as tumor necrosis factor alpha (TNF α) or suitable members of the interleukin (IL) family. Here we outline the procedure for establishing systems for studying TLR receptor activation by either gene reporter based approaches, or through stimulation of primary cells.

2 Materials

2.1 Plating HEK293 Cells

1. 0.05 % trypsin–EDTA.
2. HEK293 media: DMEM, 10 % fetal calf serum (FCS), 1 $\times$ penicillin–streptomycin.
3. 1X phosphate buffered saline (PBS): 1 mM potassium phosphate monobasic, 155 mM NaCl, 3 mM sodium phosphate dibasic, pH 7.4.
4. 96-well plate.

2.2 Trypan Blue Staining

1. 70 % Ethanol.
2. Hemocytometer.
3. Trypan Blue.
4. Handheld counter.
5. Light microscope.

2.3 Transfection and Stimulation

1. DNA stocks of required receptor, adaptor and reporter constructs.
2. 150 mM NaCl.
3. 10 $\times$ TE: 100 mM Tris–HCl, pH 7.5, 10 mM EDTA, pH 8.0.
4. jetPEI[®] transfection reagent.
5. Ligand stocks for stimulation.
6. 1 $\times$ PBS (*see item 3*, Subheading 2.1).

2.4 Luciferase Quantification

1. 1 $\times$ Passive Lysis Buffer (Promega).
2. Dual Luciferase Assay Kit (Promega).
3. Luminometer.

2.5 Bone Marrow-Derived Macrophage Culture

1. Basic BMM medium: RPMI 1640, 10 % FCS, 5 % Horse serum, 2 mM L-Glutamine, 0.05 mM 2-Mercaptoethanol, 10 µg/ml gentamycin, 1 mM sodium pyruvate (55 mg in 500 ml RPMI1640—dissolve the powder in 2–3 ml of medium and filter-sterilize).
2. Complete BMM medium: 80 % basic BMM medium, 20 % conditioned medium from L929 cells (LCM).
3. LCM: Culture supernatant from confluent L929 cells (fibroblasts producing M-CSF) grown in RPMI 1640 containing 10 % FCS and 2 mM L-glutamine. Medium is filter sterilized (pre-absorb filter with FCS) and stored at –20 °C in 40 – 50 ml aliquots. L929 cells should be initially seeded at approximately 20 % confluent.
4. 0.02 % EDTA
5. Cell scraper
6. 1× PBS (*see* **item 3**, Subheading [2.1](#)).

2.6 Stimulation of Bone Marrow-Derived Macrophages

1. Appropriate stocks of ligands for stimulation.
2. 96-well plate.
3. ELISA kits: TNFα.

3 Methods

3.1 Plating HEK293 Cells

1. Detach HEK293 cells from the surface of the flask or dish used for routine subculture (*see* **Note 1**) by incubation with pre-warmed 0.05 % trypsin–EDTA for 5 min at 37 °C.
2. Add an equal volume of pre-warmed HEK293 media, gently resuspend cells and transfer to an appropriate tube (*see* **Note 2**).
3. Count the number of viable cells using either an automated cell counter or a hemocytometer with Trypan Blue staining (*see* Subheading [3.2](#)) (*see* **Note 3**).
4. Centrifuge the cells at 1200×*g* for 5 min at room temperature.
5. Gently resuspend cells in 1× PBS (pre-warmed) then centrifuge at 1200×*g* for 5 min at room temperature.
6. Gently resuspend cells in pre-warmed HEK293 media at a density of 1.5 × 10⁵/ml.
7. Seed plates at the densities outlined in Table [1](#) to ensure an appropriate cell population for stimulation experiments 2 days later (*see* **Note 4**).

3.2 Trypan Blue Staining

1. Clean the hemocytometer with 70 % ethanol, moisten the shoulders slightly, and gently apply the coverslip.
2. Gently agitate the cell suspension (**step 3**, Subheading 3.1) and remove 100 μ l into a 1.5 ml microcentrifuge tube.
3. Add 100 μ l of Trypan Blue to the cells in the microcentrifuge tube and gently pipette up and down (*see Note 5*).
4. Carefully transfer some of the Trypan Blue stained cells to the hemocytometer by allowing it to be drawn into the chamber through capillary action.
5. Place the hemocytometer on a light microscope and focus using the 10 $\times$ objective.
6. Locate the 16 squares in one corner of the grid lines.
7. Count the number of live cells (not stained by Trypan Blue) positioned either fully within these 16 squares, or on the right-hand or bottom boundary lines.
8. Repeat **step 7** for the other three corner groups of 16 squares (*see Note 6*).
9. Take the average number of cells per 16 square grid and multiply by 2 (the Trypan Blue dilution factor). Then multiply this number by 10^4 . This will produce the cell number per ml of initial suspension.
10. Clean the hemocytometer with 70 % ethanol.

3.3 Transfection and Stimulation

1. Plate the required number of cells for the experiment to be performed at the seeding densities described in Subheading 3.1 and Table 1.
2. Prepare DNA master mixes as required (*see Note 7*). For the study of human TLR4 signaling we use the following quantities of DNA to give a total of 1 μ g DNA per ten wells: 865 ng pcDNA3, 10 ng pcDNA3:hTLR4, 5 ng pcDNA3:hCD14, 19 ng pEFIREs, 1 ng pEFIREs:hMD-2, 50 ng pNF κ B-luc, and 50 ng phRG-TK (*see Note 8*).

Table 1
Volume of media and total number of HEK cells for transient transfection assay

Plate	Volume of media	Number of cells
96-well	200 μ l	3×10^4
24-well	1 ml	1.5×10^5
6-well	3 ml	4.5×10^5

3. Add 5 μl of 10 $\times$ TE to the DNA master mix and make up to a total volume of 50 μl with 150 mM NaCl.
4. Prepare 50 μl solution of jetPEI[®] transfection reagent by adding 2 μl jetPEI to 48 μl 150 mM NaCl to give 2 μl :1 μg ratio of transfection reagent to DNA (*see Note 9*).
5. Add the 50 μl jetPEI[®] solution to the 50 μl DNA solution (*see Note 10*).
6. Vortex briefly to mix and incubate at room temperature for 30 min.
7. Add 900 μl pre-warmed HEK293 medium with antibiotics to the jetPEI[®]/DNA mix.
8. Add 100 μl of jetPEI[®]/DNA/medium mix to each well and incubate at 37 °C and 5 % CO₂ for 48 h or until stimulated.
9. After 48 h, stimulate cells for 6–8 h with an appropriate ligand (*see Note 11*).
10. Following cell stimulation, carefully remove the media from the wells and gently wash the cells with 100 μl 1 $\times$ PBS/well.
11. Remove 1 $\times$ PBS and add 50 μl 1 $\times$ Passive Lysis Buffer (*see Note 12*).
12. Incubate at room temperature for 15 min with gentle rocking.
13. Store the plate at –80 °C freezer until ready for reading of the luminescence (*see Note 13*).

3.4 Luciferase Quantification

1. Measurement of receptor activation is achieved by dual detection of Renilla and luciferase activity (*see Note 14*).
2. Thaw assay plates at room temperature.
3. Set autoinjectors 1 and 2 on the plate reader to dispense 100 μl of LARII[®] and Stop & Glo[®] reagent respectively.
4. Scrape each well of the thawed assay plate then transfer 10 μl of cell lysate to each well of a white polystyrene flat bottom 96-well plate.
5. Read plate on the luminometer using a 1–2s delay and a 5–10s read time.
6. Check the raw luciferase (LARII[®]) and Renilla readings (Stop & Glo[®]). Discard low Renilla values as these indicate cell death (<2000 on our plate reader (Fluostar OMEGA; BMG Laboratories)).
7. Divide the luciferase reading (LARII[®]) by the Renilla reading (Stop & Glo[®]) to get the relative luciferase value (Fig. 1).

3.5 Bone Marrow-Derived Macrophage Culture

1. Primary BMDM should be grown for 3 days at 37 °C and 5 % CO₂ in a total volume of 10 ml of complete media in Petri dishes.

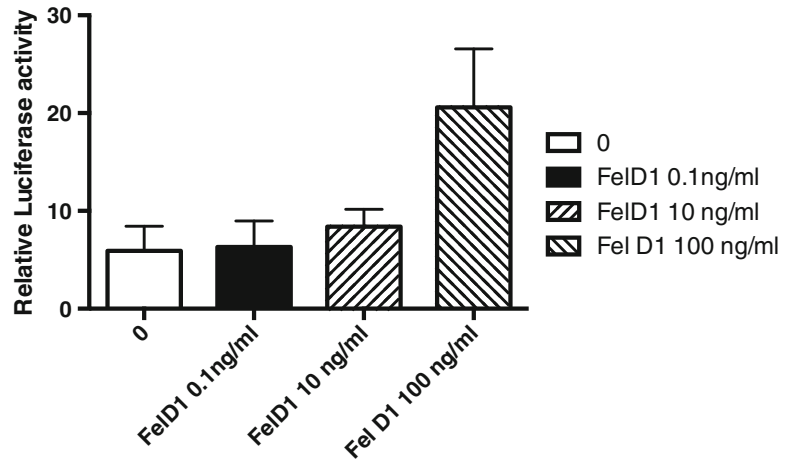


Fig. 1 A graph of the relative luciferase activity of FeID1 after addition to HEK cells transfected with TLR4, MD2, CD14, p-NfκB-Luc, and pHR-Renilla

2. On day 4 add 10 ml of pre-warmed complete medium to each BMDM-containing petri dish and incubate for an additional 2–3 days at 37 °C and 5 % CO₂.
3. On Day 6 or 7 carefully remove all medium and gently wash the cells 2–3 times using 5 ml pre-warmed basic BMM medium (*see Note 15*). Remove the media after each wash.
4. Add 10 ml of pre-warmed complete BMM medium.
5. Continue to incubate cells at 37 °C and 5 % CO₂.
6. Change the culture medium every 2–3 days until the BMDMs are confluent (*see Note 16*). At this point detach the adherent cells by gentle scraping or with EDTA and split the cells 1:3 into pre-warmed complete BMM medium in fresh culture dishes.
7. If detaching the cells using EDTA the method is as follows:
 - (a) Remove medium.
 - (b) Add 5 ml of ice-cold 0.02 % EDTA solution.
 - (c) When the cells start to detach gently pipette over the surface and collect into a 15-ml tube.
 - (d) Centrifuge at 300×g for 5 min with gentle braking.
 - (e) Remove supernatant and resuspend the cells in 6 ml of BMM medium supplemented with LCM and gentamycin.
 - (f) Add 8 ml of fresh BMM medium supplemented with LCM and gentamycin to each of three new petri dishes.
 - (g) Add 2 ml of resuspended BMDMs to each petri dish.
 - (h) Incubate at 37 °C and 5 % CO₂.

8. If using the scraping method the method is as follows:
 - (a) Remove medium.
 - (b) Add 6 ml of fresh complete BMM medium.
 - (c) Gently scrape cells off the surface of the petri dish (*see Note 17*).
 - (d) Place 8 ml of fresh complete BMM medium.
 - (e) Add 2 ml of resuspended BMDMs to each petri dish.
 - (f) Incubate at 37 °C and 5 % CO₂.

3.6 Stimulation of Bone Marrow- Derived Macrophages

1. Seed each well of 96-well plates with 2×10^5 cells (200 μ l of 10^7 cells/ml in antibiotic-free medium) one night before the experiment. Incubate at 37 °C and 5 % CO₂ in complete BMM medium.
2. Add 20 μ l of ligand to each well at an appropriate dilution (*see Note 18*). Use 20 μ l 1 $\times$ PBS as a negative control ligand.
3. Incubate at 37 °C and 5 % CO₂ for 2 h.
4. Transfer 50 μ l of culture supernatant to a fresh 96-well plate and store this at -80 °C for TNF α detection (*see Note 19*).
5. Remove rest of the culture medium and add 200 μ l of ligand free complete BMM medium.
6. After 4–6 h (7–9 h after addition of stimulant), transfer 50 μ l of culture supernatant to a fresh 96-well plate and store this at -80 °C for TNF α detection (*see Note 20*).

4 Notes

1. Cell passage number should be similar for experiments that are to be compared with one another. As the passage number of HEK293 cells increases the cells often become less responsive to stimulation. In general aim to use cells with a passage number less than 30.
2. For a T75-flask use 3–5 ml of 0.25 % trypsin–EDTA and resuspend cells using cell culture media to a total volume of approximately 10 ml prior to transfer to a 15 ml Falcon tube.
3. Accurate determination of cell number is crucial for accurate and reproducible cell seeding and experimental reproducibility.
4. If seeding for experimentation 3 days later (i.e., seeding on a Friday, begin experiment on a Monday) then use 1.5×10^4 cells/well (200 μ l 7.5×10^4 ml⁻¹) for a 96-well plate.
5. Do not over pipette or vigorously mix the cells as this will lead to cell lysis.

6. If cell viability data is also wanted then additionally count the number of dead cells that are Trypan Blue positive.
7. The following plasmid amounts are for 96-well plates, other sizes of culture vessel will need scaling appropriately.
8. The precise plasmids will depend on the reporter system being used and the signaling pathway being studied. The example here is for dual luciferase reporters of TLR4 signaling. Total DNA concentration is bulked with empty vector plasmids, e.g., pcDNA3 and pEFIREs. To determine whether allergens activate other TLRs we use 10X the DNA concentration of the receptor plasmid because TLR4 shows high levels of basal activity when over expressed.
9. Manufacturers recommend trying two ratios of jetPEI to DNA. Firstly, 2 μ l jetPEI[®]–1 μ g DNA and secondly 3.2 μ l jetPEI[®]–1 μ g DNA. For a 2 μ l–1 μ g ratio add 2 μ l jetPEI to 48 μ l NaCl; and for a 3.2 μ l–1 μ g ratio add 3.2 μ l jetPEI to 46.8 μ l NaCl. We have found no difference between the two ratios.
10. The jetPEI[®] solution must be added to the DNA solution, not the other way round.
11. Stimulating ligands should be prepared in media containing 0.1 % FCS. Whilst the precise composition and concentration of these stimuli will depend on the system being studied examples for the stimulation and/or study of TLR4 signaling include. Whenever assay conditions are changed, including the use of different cells or plasmids, a dose response for the ligands should be performed to ensure use of the optimal concentration. For HEK293 based reporter assays we have previously used the following concentrations of stimulatory ligands [5, 11]: Lipoteichoic acid (TLR2) 10 ng/ml; Lipid A (TLR4) 10–100 ng/ml; Lipid IVa (TLR4) 1 μ g/ml; LPS (TLR4) 1–100 ng/ml; Fel d 1 (TLR4) 0.1–100 ng/ml; Flagellin (TLR5) 5–50 ng/ml.
12. Prepare from stock diluted in 1 $\times$ PBS. The Passive Lysis Buffer is formulated to minimize background auto-luminescence.
13. Ensure that the 96-well plate containing the samples for analysis is suitable for use in luminescence studies. A freeze thaw step enhances the efficiency of cell lysis.
14. We use the Dual Luciferase Assay from Promega. Best results are obtained if the samples are equilibrated to room temperature.
15. This will remove non-adherent cells and transiently attached cells.
16. BMM cultures may not become as full in petri dishes as macrophage cell-lines. Watch the cultures to see if cells start to detach from the plastic.

17. Gently pipette cell suspension up and down a few times to reduce clumping. Do not pipette over-vigorously and avoid any foaming.
18. The concentration of ligand used will vary with experimental setup and nature of stimuli. As examples we have used: lipoteichoic acid (TLR2) 50 ng/ml; PAM3CSK4 (TLR2) 1 ng/ml; LPS (TLR4) 0.05–0.5 ng/ml; Fel d 1 (TLR4) 25–100 µg/ml. If infecting with bacterial suspensions (i.e., *Salmonella*) then 20 µl of the appropriate concentration of bacteria (resuspended in 1× PBS) to provide the desired multiplicity of infection should be added to each well containing macrophages.
19. If other cytokines are to be measured then remove suitable volumes of supernatant for downstream analysis by ELISA or other suitable approach. Detection of TNFα works well as a positive control.
20. Remove additional volumes of supernatant as required for cytokine targets. 200 µl fresh medium can be added and incubation continued for the desired period of analysis.

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