NF-κB Transcriptional Activity Assay using NKL reporter cells

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Method Article

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Abstract

NF-κB is a transcription factor that regulates diverse biological and cellular responses. NF-κB activation is triggered by many stimuli and kept in check at multiple steps. Further, transcriptional activity of NF-κB is modulated by various post-translational modifications. Therefore, direct measurement of NF-κB transcriptional activity utilizing reporter assay system is critical for the direct analysis of NF-κB pathway activation. Here we describe step by step protocol to measure NF-κB transcriptional activity in NK cells using stable NKL cell lines transduced with lentiviral κB-GFP construct.

Introduction

NF-κB is involved in various transcriptional programs induced by variety of cellular processes, such as cell proliferation, survival, differentiation, stresses, and in particular immune and inflammatory responses. And its aberrant regulation is implicated in diverse diseases including cancer, metabolic disorders, autoimmune or inflammatory diseases. Therefore, the signaling pathway leading to the activation of NF-κB is controlled at multiple levels through elaborate positive and negative regulatory components. Further, optimal NF-κB activation is also modulated by post-translational modifications of NF-κB components, including the p65, via the phosphorylation, acetylation, ubiquitination, and so on. Crosstalk with other transcription factors, such as IRF3, STAT3, c-Jun, and c-Fos, can also influence the activity of NF-κB. As a result, direct analysis of the transcriptional activity of NF-κB is indispensable for the study of NF-κB activation pathway. To this end, we generated stable NKL reporter cell lines that express GFP under the control of NF-κB transcription response element \(TRE), instead of transient transfection of reporter vector. We have selected transduced cells with selective antibiotics and further isolated the responding cells by FACS sorting. Measurement of GFP expression following NF-κB-activating stimuli enables direct single cell-based FACS analysis of NF-κB activation, instead of commonly used indirect assays \(e.g. phosphorylation of NF-κB components, degradation of IκBα, nuclear translocation and DNA binding activity of p65). For the stimulation of NKL reporter cells, we immobilized NK cell receptor-specific antibodies onto high-binding flat-bottom 96well plate. This protocol describes detailed steps of constructing reporter cells and the analysis of NF-κB transcriptional activity of NK cells following receptor stimulation.

Reagents

Cell lines: 293TN packaging cell line \(System Biosciences), NKL 293TN culture medium: DMEM \(Cellgro) supplemented with 10% heat-inactivated FBS \(Cellgro) Lentivirus harvesting medium: DMEM supplemented with 10% heat-inactivated FBS and 11mg/ml BSA \(Sigma) NKL culture medium: RPMI1640 \(Cellgro) supplemented with 10% heat-inactivated FBS, 1mM sodium pyruvate \(Gibco), and 200U/ml recombinant human IL-2 \(rIL-2, Roche) NKL resting medium: RPMI1640 supplemented with 5% heat-inactivated FBS, 0.5mM sodium pyruvate, without rIL-2 6well plate, 12well plate \(Thermo) OptiMEM \(Gibco) pGreenFire-NF-κB-EF1-Puro vector \(System Biosciences) pPACKH1 packaging plasmid mix \

(System Biosciences) XtremeGENE 9 transfection reagent \(Roche) Polybrene \(Calbiochem) Puromycin \(Calbiochem) High-binding flat-bottom 96well plate \(Costar)

Equipment

Standard equipment for cell culture FACS Aria \(BD biosciences) FACS Accuri \(BD biosciences)

Procedure

Generation of NKL reporter cells by lentiviral transduction 1. Plate 0.5X10^6 293TN cells/well into 6well plate with 2ml of DMEM \(10% FBS) and culture in 37°C CO_2 incubator overnight 2. Prepare transfection mix: OptiMEM 150µl + XtremeGENE9 6µl Flick and incubate for 5min at room temperature Add pGreenFire-NF-κB-EF1-Puro 1.67µg + pPACKH1 1.67µg Flick and incubate for 30min at room temperature 3. Transfer transfection mix dropwise into medium with cultured 293TN cells and incubate for 18hr in 37°C CO_2 incubator 4. Change medium with 2.5ml DMEM \(10% FBS, 11mg/ml BSA) and incubate for 24hr in 37°C CO_2 incubator 5. Gently harvest 2ml of supernatant \(1st harvest) and store at 4°C 6. Add another 2.5ml DMEM \(10% FBS, 11mg/ml BSA) and incubate 24hr in 37°C CO_2 incubator 7. Gently harvest 2ml of supernatant \(2nd harvest) and pool with 1st harvest 8. Centrifuge virus-containing supernatant pool at 1250rpm for 5min to exclude debris. 9. Add 10µg/ml polybrene and 200U/ml recombinant human IL-2 to virus-supernatant 10. Resuspend 0.5X10^6 NKL cells \(2 days after split) with 2ml of virus-supernatant, transfer to 12 well plate and incubate for 10min in 37°C CO_2 incubator 11. Seal the plate with parafilm and centrifuge at 700g for 30min at 32°C \(1st spin-infection) 12. Remove parafilm and incubate for 3hr in 37°C CO_2 incubator 13. Seal the plate with parafilm and centrifuge at 700g for 30min at 32°C \(2nd spin-infection) 14. Remove parafilm and incubate for 4hr in 37°C CO_2 incubator 15. Centrifuge at 1400rpm for 5min to remove supernatant and resuspend transduced NKL cells with 10ml of NKL culture medium \(RPMI1640, 10% FBS, 1mM sodium pyruvate, 200U/ml IL-2) 16. Transfer cell suspension to T25 flask and incubate in 37°C CO_2 incubator 17. At 2 days after infection, add 1µg/ml puromycin 18. At 3 days after infection, centrifuge cells at 1400rpm for 5min and resuspend with 10ml of NKL culture medium with 1µg/ml puromycin and transfer to T25 flask 19. Maintain transduced cells with NKL culture medium containing 1µg/ml puromycin by splitting cells with 3days interval 20. Further select transduced NKL cells showing upregulation of GFP after TNF-α \(10ng/ml) treatment by sorting with FACSAria **NF-κB reporter assay with NKL reporter cells** 1. Prepare 5µg/ml stimulating antibodies specific for NK receptors in PBS 2. Pipette 100µl/well in flat-bottom high-binding 96well plate 3. Cover plate with sealing tape and incubate overnight at room temperature 4. Resuspend NKL cells, those are rested for 24hr in NKL resting medium \(RPMI1640, 5% FBS, 0.5mM sodium pyruvate, without IL-2) following 2days culture after split, with NKL resting medium at 2X10^6 cells/ml 5. Empty plate contents and wash wells 3 times with 240µl/well of PBS 6. Pipette resuspended NKL cells 100µl/well in antibody-immobilized plate 7. Cover plate with lid and incubate for 6hr in 37°C CO_2 incubator 8. Place the plate on ice, harvest from each wells and transfer cells into each 1.5ml eppendorf-

tubes 9. Place 1.5ml eppendorf-tubes on ice and add 500µl/sample of FACS buffer \(DPBS, 1% FBS) 10. Centrifuge at 2000rpm for 5min at 4°C and remove supernatant 11. Resuspend cells with 200µl of FACS buffer and analyze the upregulation of GFP with FACS Accuri