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Abstract

This protocol contains instructions on how to conduct molecular biology experiments, including transfection and reporter assays, RNA extraction and qPCR, coimmunoprecipitation and immunoblot analysis, viral plaque assays, silver staining and mass spectrometry analyses, recombinant protein purification and *in vitro* GST pull-down assays, *in vitro* tyrosine phosphorylation assays, CRISPR/Cas9 knockout, confocal microscopy, native PAGE, and preparation of cytoplasmic and nuclear proteins. How to isolate primary murine pDCs. How to evaluate the oncolytic effect of SVA using a xenograft tumor model.

Troubleshooting

Transfection and reporter assays

1 The cells were transfected by standard calcium phosphate precipitation or Lipofectamine 2000. To normalize the transfection efficiency, pRL-TK (*Renilla*luciferase) reporter plasmid (10 ng) was added to each transfection. To ensure that each transfection received the same amount of total plasmid DNA, the empty control plasmid was added to each transfection. Twenty-four hours after transfection, cells were treated or untreated with the indicated stimuli before luciferase assays were performed using a dual-specific luciferase assay kit. Firefly luciferase activities were normalized on the basis of *Renilla* luciferase activities.

RNA extraction and qPCR

2 Total RNA was isolated from cells using RNAiso plus reagent. After reverse-transcription with HiScript II Q RT SuperMix, the cDNA was diluted 50-fold and subjected to qPCR analysis to measure mRNA levels of the tested genes. Data shown were the relative abundance of the indicated mRNAs normalized to that of *GAPDH*.

Coimmunoprecipitation and immunoblot analysis

3 Cells were lysed in l mL NP-40 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40) supplemented with protease and phosphatase inhibitors. The lysates were centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was divided into two equal portions and incubated with 30 mL Protein G sepharose beads and the indicated antibody or control IgG (1 mg) at 4 °C for 3 h. Beads were then washed three times with lysis buffer containing 0.5 M NaCl. The precipitates were resuspended in 2 × SDS loading buffer (50 mL), boiled for 15 min, and further analyzed by standard immunoblot procedures.

Isolation of primary murine pDCs

4 Spleens were obtained from 2-month-old C57BL/6J background mice. A suspension of single splenocytes was obtained by passage of the spleens through a 40-mm-mesh-size cell strainer after lysis of red blood cells with ACK lysis buffer. Splenic pDCs were purified by negative magnetic selection using the Mouse Plasmacytoid Dendritic Cell Isolation Kit. pDCs were then infected with VSV or HSV-1 for the indicated times before qPCR and immunoblot analysis.

In vivo SVA efficacy

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Athymic female mice (BALB/c-nu), aged 6-8 weeks, were injected subcutaneously in the flank with wild-type or BLK-KO U87MG cells (5×10^6 cells per mouse) in 10% phenol red-free Matrigel. Once tumors reached volumes of approximately 100 mm^3 (~ 7 days), mice within each cohort were randomly distributed and challenged with SVA (1×10^8 PFU per mouse) or an equivalent volume of PBS, pH 7.4, via intravenous injection.

Twenty-four hours after SVA challenge, tumors (n = 7 for each group) were excised, and the same mass of tumor tissue was homogenized for 10 s in PBS. The suspensions were centrifuged at 1,620 g for 10 min, and the supernatants were collected for ELISA measurement of the indicated cytokine levels according to the manufacturer's instructions.

Three days after SVA challenge, tumors (n = 4 for each group) were excised, and the same mass of tumor tissue was homogenized for 10 s in PBS. The suspensions were centrifuged at 1,620 g for 10 min, and the supernatants were subjected to viral plaque and qPCR assays, respectively.

For *in vivo*antitumor efficacy studies, tumors (n = 5-6 for each group) were measured in two dimensions with Vernier calipers every 2 days, and tumor volumes were calculated using the formula $V = (L \times W^2)/2$, where L is the length or diameter and W is the width. Each data point represents the average tumor volume with error bars representing the SD and was plotted using GraphPad Prism 6 software. At the end of the study, mice were euthanized by CO_2asphyxiation.

Viral plaque assays

6 Eight week-old mice were infected with VSV for 4 days, the spleens of mice were weighted and homogenized for 10 s in PBS. After homogenization, the spleen suspensions were centrifuged at 1,620 g for 10 min, and the supernatants were collected for plaque assays. Vero or IBRS-2 cells were seeded in 24-well plates, and the cells were infected with serial dilutions of the spleen or tumor tissue supernatants at 37 °C for 2 h, overlaid with 2% methylcellulose and further incubated for 36-48 h. The overlay was

then removed, and the cells were fixed with 4% paraformaldehyde for 30 min and stained with 1% crystal violet for 30 min before plaque counting.

Silver staining and mass spectrometry analyses

7 HEK293 cells (2×10^7) were transfected with GST-BLK or GST plasmids for 24 h. Cells were then infected with SeV (MOI, 1) for 4 h before treatment with NP-40 lysis buffer containing protease and phosphatase inhibitors. GST-BLK-associated factors were immunoprecipitated from lysate supernatant using glutathione sepharose beads. Immunoprecipitated proteins were detected by immunoblot followed by silver staining. The gel bands were separated and further analyzed by mass spectrometry.

Recombinant protein purification and in vitro GST pull-down assays

8 Recombinant GST-BLK, GST-IRF3 and His-IRF3 proteins expressed in *E. coli* Rosetta strain were purified with glutathione sepharose or Ni-NTA resin by the AKTA protein purification system. TBK1-Flag and IRF3-Flag proteins expressed in HEK293 cells were purified with Protein G sepharose beads and eluted from beads with 3×Flag peptide (1 mg/mL). Then, His-IRF3, TBK1-Flag, or IRF3-Flag protein was added to the purified recombinant GST-BLK protein coupled to glutathione sepharose beads and incubated for 3 h at 4 °C. Subsequently, the beads were washed and boiled. The eluates/inputs were fractionated by SDS-PAGE and detected by Coomassie staining or immunoblot analysis.

In vitro tyrosine phosphorylation assays

9 Recombinant GST-IRF3 protein expressed in *E. coli* Rosetta strain was purified with glutathione sepharose and eluted from beads with elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione). For *in vitro* kinase assays, vector, Flag-tagged BLK and its mutant plasmids were individually transfected into HEK293 cells for 24 h. These proteins were then purified with Protein G sepharose beads and co-incubated with purified GST-IRF3 (50 mg) in an equal volume of 2 × reaction buffer (100 mM Tris-HCl, 20 mM $MgCl_2$, 1 mM Na_3VO_4, 4 mM DTT, pH 7.2), and 1 mM ATP was added prior to incubation for 1 h at 30 °C. The reaction was stopped by adding 1/5 volume of 6 × SDS loading buffer and boiled for 15 min at 95 °C before immunoblot analysis. For BLK autophosphorylation assays, vector, Flag-tagged BLK and its mutants plasmids were individually transfected into HEK293 cells for 24 h. These proteins were then purified with Protein G sepharose beads and incubated with 50 mL of 1 × reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM Na_3VO_4, 2 mM DTT, pH 7.2), and 1 mM ATP was added prior to incubation for 1 h at 30 °C. The reaction was stopped by adding 1/5 volume of 6

× SDS loading buffer and boiled for 15 min at 95 °C before immunoblot analysis.

CRISPR/Cas9 knockout

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Double-stranded oligonucleotides corresponding to the target sequences were cloned into the lentiCRISPR v2 vector. The constructed plasmid (10 mg) was then co-transfected with two packaging plasmids (LH1 (7.5 mg) and LH2 (5 mg)) into HEK293 cells. The culture medium was replaced with fresh medium without antibiotics 12 h after transfection. After an additional 36 h, the medium containing lentiviral particles was filtered (0.22 mm filter, Millipore) and used to infect Jurkat, Raji, U87MG or A20 cells in the presence of polybrene (8 mg/mL). The infected cells were selected with puromycin (1 mg/mL) or blasticidin S (10 mg/mL) for at least 6 days before additional experiments were performed.

Confocal microscopy

11 Cells were infected with SeV for the indicated times, then fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min at 4 °C. Cells were blocked with 1% BSA in PBS and stained with the indicated primary and secondary antibodies for 2 h. The nuclei were stained with DAPI. Cells were observed with Nikon confocal microscope under a 60× oil objective. Quantitative analysis of colocalization images was performed using the open source Fiji (ImageJ) software.

Native PAGE

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Control or virus-infected cells were collected with ice-cold PBS and lysed with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, pH 7.4) containing protease inhibitors. The lysates were centrifuged at 12,000 rpm for 15 min. The supernatants were then subjected to native PAGE on an 8% gel without SDS. The gels were pre-run with running buffer (25 mM Tris-HCl, 192 mM Glycine, pH 8.4, with or without 0.2% deoxycholate in the cathode and anode buffer, respectively) at 75 V for 30 min. Subsequently, the samples were electrophoresed at 75 V for 3 h at a cold temperature and further transferred onto NC membranes for immunoblot analysis.

Preparation of cytoplasmic and nuclear proteins

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The assays were performed with a NE-PER nuclear and cytoplasmic extraction reagent kit according to the manufacturer's instructions. Briefly, SeV- or HSV-infected cells were collected with ice-cold PBS and lysed by blowing 30 times with a 1 mL syringe in 500 mL membrane lysis buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.4% NP-40, pH 7.9) containing protease inhibitors. The homogenate was centrifuged at 500 g for 10 min. The supernatant was saved as cytosol, and the pellet was saved as crude nuclei. The crude nuclei were washed twice with 500 mL membrane lysis buffer and resuspended in 20-50 mL of extract buffer (20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, pH 7.9) and shaken vigorously every 30 s for 15 min, followed by centrifugation at 12,000 rpm for 10 min. The supernatants containing nuclear or cytoplasmic proteins were subjected to immunoblot analysis.