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Cell Culture and estimation of cytokines by ELISA

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Mohd Khubaib¹

¹University of Hyderabad



Mohd Khubaib

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Protocol status: In development

We are still developing and optimizing this protocol

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Abstract

this protocol describes the maintainance and treatment of RAW 264.7 macrophage cell lines with Mycobacterial PE/PPE proteins. It also describes estimation of secreted cytokines through ELISA. Estimation of level of IgG subclass from the serum is also described here.



Materials

MATERIALS

 Cytokine ELISA kit **Becton Dickinson (BD)**

 Peprotech ELISA kit **peprotech**

 DMEM **Invitrogen - Thermo Fisher**

 FBS **Invitrogen - Thermo Fisher**

- 1 RAW264.7 cells were seeded (0.1 million/well) in 96-well plate. Cells were stimulated with different concentration of native or heat inactivated recombinant protein. After 24hrs of stimulation, culture supernatant was harvested and ELISA was performed as described earlier. Splenocytes from immunized and placebo treated mice were also seeded in 96-well plates (0.1 million/well) in DMEM. Protein treatments with different concentration were given for 72 hours and supernatants were collected and stored at -20°C.
- 2 B-cell response against individual proteins was carried out using specific secondary antibody against IgG1, IgG2a and IgG2b. Blood was collected by bleeding mice via the lateral tail vein. Sera were collected and stored at -20°C to be used later for ELISA as described earlier. Briefly, 96 well plates were coated by specific proteins in PBS (10µg/ml) and kept at 4°C overnight. Plate was washed 3 times with wash buffer and blocked for an hour at room temperature. After 3 washes serum samples in 1:100 dilution was added and kept for 2 hours. Secondary conjugate antibody was added in 1:5000, 1:3000 and 1:3000 respectively for IgG1, 2a and 2b for an hour. Plate was washed at least 5 times, TMB substrate was added, and reaction was stopped with 2N H₂SO₄.
- 3 Secreted cytokine levels in culture supernatant were measured using OptEIA kits (BD Biosciences, San Diego, CA) to determine the levels of mouse TNF-α, IFNγ, IL-10, IL-6. Peprotech (Rocky Hill, NJ) standard ELISA development kits were used to estimate the level of Human TNF-α and IL-6. ELISA was carried out using manufacturer protocol or as described earlier. Briefly, 96 well ELISA plates were coated with capture antibody in coating buffer (bicarbonate/phosphate buffer) kept at 4°C overnight. Plates were washed with PBS-T (0.05% tween20) thrice. 10% FBS was used as blocking as well as assay diluent. After an hour of blocking, supernatant along with standards were added for 2 hrs. After 5 washes, detection antibody and enzyme conjugates were added for 1hr. After 7 washes, TMB substrate was added and 2N H₂SO₄ was added to stop the reaction. Absorbance was taken at 450nm and curve was plotted along with standards to determine the cytokine levels in test samples.