STIMULATION & CULTURING OF HEK293TS STABLY EXPRESSING MNOD2 & SEAP-NFκB REPORTER

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BACKGROUND:

This cell line is originally derived from human embryonic kidney (HEK293) cells grown in tissue culture. The HEK293T variants contain the SV40 Large T-antigen, which allows for episomal replication of transfected plasmids containing the SV40 origin of replication. In comparison to HEK293s, HEK293Ts are more easily transfectable. This particular cell line has been stably transfected to express mNod2, an intracellular PRR that detects bacterial peptidoglycan-derived muramyl dipeptide (MDP), and the SEAP-NFκB Reporter. SEAP is the secreted form of human embryonic alkaline phosphatase. Upon recognition of a PAMP, Nod signaling leads to the downstream activation of NFκB, a transcription factor that induces a pro-inflammatory response. In these cells, activation of NFκB leads to the expression of SEAP, which can be detected through the use of HEK Blue Detection Media (Invivogen).

CULTURING:

- This cell line grows at a slower rate than regular HEK293Ts. Split cells when approximately 80-90% in confluency
- Keep cells in 20mL of Complete DMEM (10% FBS, 5mL Pen-Strep, 5mL L-glutamine) and under constant antibiotic selection → 50uL of 10mg/mL Zeocin & 100uL of 1mg/mL Blasticidin
- Work in a biosafety hood in a tissue culture room. Ensure sterility by spraying everything with 70% ethanol

Splitting Procedure:

1. Discard medium by pipetting from flask into waste container

2. Rinse cells with 5mL PBS (heated to 37°C) to remove all remaining traces of media. Remove PBS.

3. Add 10mL of Complete DMEM (37°C, containing antibiotics) and use pipette to lift cells off flask wall.
   Note: HEK293Ts are loosely adherent; trypsin can be used if there is difficulty lifting

4. Aliquot 19mL of medium to a 600mL flask. Add 1mL of cell suspension to each flask. Mix gently.

5. Label flask(s) with cell line, passage number, date, and name. Place in 37°C incubator.

Detection of MDP in Mouse Serum

EQUIPMENT & MATERIALS:

- Level 2 Bio Hood, Tissue Culture Room (MDCL 4097)
- 37°C, 5% CO_2 Incubator, Tissue Culture Room (MDCL 4097)
- Vortex, Tissue Culture Room (MDCL 4097)
- Hot Plate (MDCL 4077)
- Spectramax Plate Reader (CMCB, MDCL 2nd floor)
  Note: any plate reader will do that can read absorbance at 630nm

- Complete DMEM (10% FBS, 1% L-glut & Pen-Strep)
- HEK293T line stably expressing mNod2 & SEAP-NFkB (P<10)
- HEK Blue Detection Media (Invivogen), heated 37°C in water bath
- Mouse serum (*minimize freeze-thaw cycles*)
- Phosphate-Buffered Saline
- Endotoxin-free H_20 (autoclaved MilliQ water works as well)

PROTOCOL:

DAY 1: CELL PREPARATION

1. Seed 4000 cells/well in a 96-well format in a volume of 200uL in Complete DMEM media
Seeding needs to be done ~ 24 hours before beginning the assay to give cells enough time to adhere.

DAY 2: SAMPLE PREPARATION & STIMULATION

1. Dilute serum samples 1:5 in PBS, then 1:1 in sterile water (autoclaved MilliQ water works). *The dilution will be 1:10 overall. For this assay, I aim for 1uL of serum per well and perform the assay in triplicate.*

2. Heat-inactivate samples at 75°C for 5 min.
The purpose of the previous dilution was to allow for proper heat inactivation without impairing the integrity of your samples. I have tried MANY different heat inactivation temperatures, dilutions, and proteinase-K/β-mercaptoethanol treatments. This method was found to work best. If you do not dilute your samples, the serum can adopt a gel-like consistency. If you do not HI your samples, clumping will occur in wells.

3. Add 10uL of diluted sample & 190uL of HEK Blue Detection media (per well) into appropriately labeled tube.
Each 10ul is 1ul of your sample. If you're doing duplicates, add 20uL sample + 380uL HBD media per tube. You can try different volumes.

4. Once samples are prepared, discard ALL old media from 96-well plates, and then add

200ul of your different sample solutions. Incubate the plate(s) in the 37°C, CO_2 Incubator.
To save time, I used a multichannel pipette to discard old media. Try your best to minimize the amount of time that wells are free of media i.e. remove media from one section of the 96-well plate at a time.

DAY 3: ABSORBANCE SPECTROSCOPY FOR CALORIMETRIC DETECTION OF MDP

1. Use a plate reader to read your plates at absorbance of 630nm.
You may set your negative control (HBD media alone) as your blank to ease analyzing the data – there usually is background SEAP expression. Samples with readings close to and below 0 will be designated as below detection limit.

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Notes:

♦ For each plate, you may create a standard curve if you wish to know the concentration of MDP in your samples (0 to 0.5ug/mL). If you just want to know relative differences in your samples, you may just use positive and negative controls, **positive** being 0.5ug/mL of MDP & **negative**, the HEK Blue Detection media alone and/or gnotobiotic serum.

♦ If you wish to transfect in serum, you will get a more robust response. However, the assay is responsive enough to add serum directly into media without transfecting. Furthermore, opting not to transfect would better emulate the biological response *in vivo*.

♦ Literature suggests that acidification of media aids in cellular uptake of MDP

Example Data:

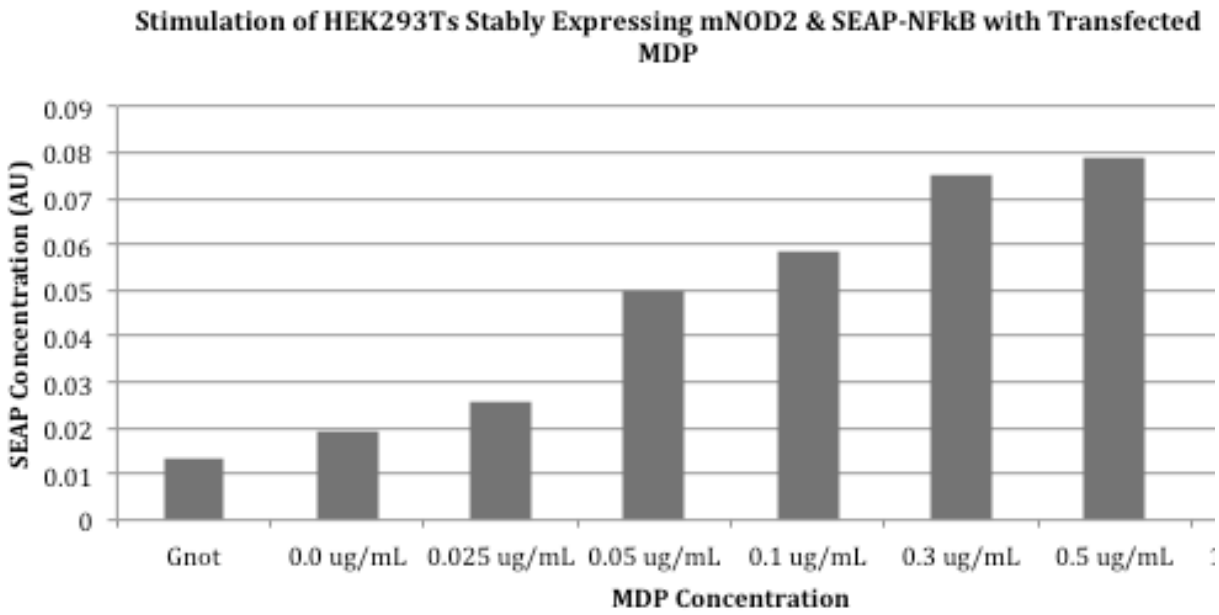
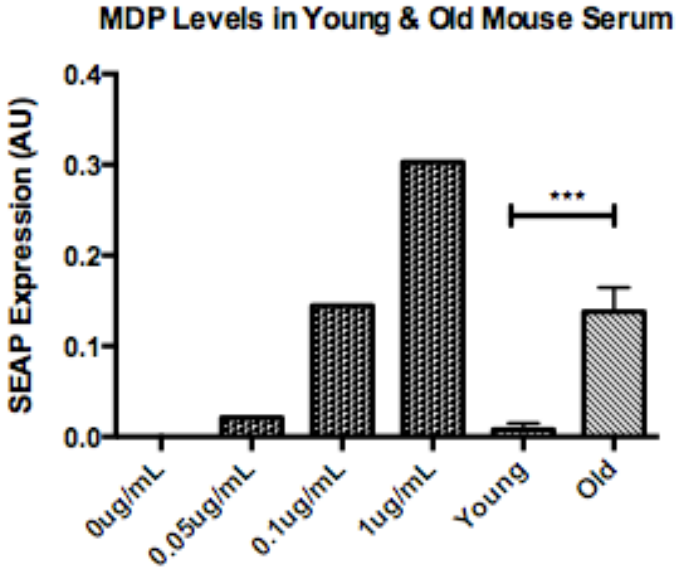


Fig. HEK293T cells stably expressing mNod2 & a SEAP-NFkB reporter (P=6, 90% viability) were seeded at $1x10^4$ cells per well in a 24-well plate. Twenty-four hours following seeding, cells were transfected with various concentrations of MDP using PEI. Gnotobiotic serum (0.5ul per well), in the absence of MDP, was used as a negative control. Cells were read at 630nm on the *Spectramax* 18 hours following transfection.



Data from this figure was attained following the above protocol (page 2) exactly. Young and old bars represent averages of groups of mice.

Expression was read relative to 0ug/mL.