Supplementary Protocols:


Protocol S2. Phase I protocol for THP-1 cells.


Protocol S4. Phase II protocol for THP-1 cells.
SUPPLEMENTARY MATERIALS

Protocol S1

Dispersion protocols

Preparation of NP suspensions for in vitro experiments

All the NP solutions are prepared fresh from stock solutions (e.g. 10 mg/ml). The stock solutions are made up from dry powder unless already in solution. Depending on the volume concentration of particles in solution, an appropriate volume of WFI grade H₂O is added to the appropriate volume of particles. Dry powder NP are weighed using a sensitive scale (e.g. Mettler Toledo Cat.# AL54) that can weigh µg quantities of material. The powder is then brought to a concentration equal to 10 mg/ml using cell WFI grade H₂O (Irvine Scientific, Cat.# 9309). For cell culture experiments this stock is vortexed (or sonicated using Sonicating Water Bath) immediately before diluting into complete cell culture medium (that contains 10% fetal calf serum). For instance, a 50 µg/ml exposure dose is made by adding 5 µl of the NP into 1 ml of complete culture medium. The NP suspension is then sonicated for 10 seconds prior to cellular exposure assays.

Titanium dioxide nanobelts (TNB) dispersion protocol for in vitro experiments

Materials used:

- Stir bar           Fisher Scientific cat# 14-512-150
- 1.8 ml glass vials Fisher Scientific cat# 03-339-25A
- 7.5% BSA in DPBS   Sigma cat# A8412-100ML

- Weigh out approximately 5 mg of TNB (batch #A8) on a reliable analytical balance such as ones made by Sartorius or Mettler.
- Record the exact mass and place the TNB in the autoclaved glass vial with the autoclaved stir bar already inside.
• Under a sterile hood, add the appropriate amount of 7.5% BSA solution to create a 5 mg/ml suspension. This can be done by the following formula:
• Recorded mass in mg ÷ 5 mg = the volume in ml to add to the glass vial (It should be close to 1.0 ml)
• After the volume has been added cap the glass vial with an autoclaved plastic cap and place the vial on a magnetic stir plate for one hour at a gentle speed setting. You may have to gently vortex the vial to get the stir bar to seat correctly at the beginning. Make up fresh for each experiment. You can do a gentle vortex before use to re-establish the suspension. **Do not** sonicate the TNB suspension, as they will break into smaller irregular rods with differing bioactive properties.

**Preparation of MWCNTs suspensions**

**Heat inactivated serum should be used to prepare nanoparticle suspensions to decrease the background in LDH assays.**

• All the MWCNTs solutions are prepared fresh from stock solutions (5 mg/ml).

• To make Stock solutions, dry powder MWCNTs are weighed using a sensitive scale (e.g. Mettler Toledo Cat.# AL54) that can weigh mg quantities of material.

• The powder is then brought to a concentration equal to 5 mg/ml using cell WFI grade H₂O (Irvine Scientific, Cat.# 9309).

• For cell culture experiments this stock is vortexed (pulse for 5 second) then sonicated for 15 min using Water Bath Sonicator (Branson, Model 2510, 100 W output power; 42 kHz frequency) immediately before diluting into complete cell culture medium (BEGM contains 600 µg/mL BSA and 10 µg/mL DPPC; RPMI contains 10 % heat-inactivated FBS).

• Make the highest concentration suspension first. For instance, a 100 µg/ml exposure dose is made by adding 20 µl of the NP into 980 µl of complete culture medium.

• Sonicate the highest concentration suspension using a probe sonicator (Sonics and Materials Inc., Model vcx130, 130 W output power; 20 kHz frequency) at 40 % amplitude for 15 s followed by 15 s break, then another 15 s sonication.

• After sonication, make lower concentrations by making dilutions from the highest concentration. Vortex all suspensions again before cell treatment. The diluted NPs are inverted 3-4 times before each use to ensure that they stay well mixed.
5. Stability of MWCNTs suspensions

- The stability of the freshly prepared NP suspensions at 50 µg/mL is tested by light absorbance (UV-Vis) at 550 nm at 0, 3 and 24 hr after sonication and all measurements are done at room temperature.
- The stability of the freshly prepared NP suspensions can also be followed by dynamic light scattering (DLS) at 0, 3 and 24 hr after sonication (optional if the equipment is not available) and all measurements are done at room temperature.

6. Starting dosages:

- Selected dose range for ZnO is 4, 10, 25, 50 µg/ml.
- Selected dose range for MWCNTs suspension are 10, 25, 50,100 µg/mL.

Assessment of MWCNT Suspension Stability Index. A kinetic analysis of the suspension stability of AP-, PD- and COOH-MWCNTs in PBS was performed by monitoring the supernatant absorbance at 550 nm for different lengths of time, using UV-vis spectrometry (SpectroMax M5e, Molecular Devices Corp., Sunnyvale, CA). Typically, 1 mL of suspension containing 50 µg/mL MWCNT was prepared with or without dispersing agents as described in the Methods and Materials section. Absorbance readings were taken at set time intervals over 24 hr. The stability suspension index was calculated by expressing the absorbance in the supernatant as a % of the absorbance at t = 0.

Phase I protocols:

Cell culture Protocols for BEAS-2B, RLE-6TN Cells

Culture of BEAS-2B Cells

BEAS-2B cell culture (transformed cells): a human bronchial epithelial cell line was obtained from Lonza, Inc. (Walkersville, MD). All cell strains tested negative by PCR for HIV-1, hepatitis B and hepatitis C. Catalog number: CRL-9609 (Lot# 58121836)

Materials

1. CC-3170 BEGM BulletKit: Kit contains a 500 ml bottle of BEBM (CC-3171) and BEGM SingleQuots (CC-4175) containing Supplements and Growth factors (BPE,
hydrocortisone, hEGF, epinephrine, insulin, triiodothyronine, transferrin, gentamicin/amphotericin-B and retinoic acid.

2. Trypsin 0.05% (1x) with EDTA 4Na (Gibco 25300-054)
3. Dulbecco’s PBS (ATCC 30-2200 or Gibco 14190-144)

**Preparation of Media:**

For BulletKits®, perform the following steps:

1. Decontaminate the external surfaces of all supplement vials and the medium bottle with ethanol or isopropanol.
2. Aseptically open each supplement vial and add the entire amount to the basal medium with a pipette.
3. Rinse each cryovial with the medium. It may not be possible to recover the entire volume listed for each cryovial.
4. Note: If there is concern that sterility was compromised during the supplementation process, the entire newly prepared growth medium may be refiltered with a 0.2 µm filter to assure sterility. Routine refiltration is not recommended.

**Thawing of Cells/Initiation of Culture Process:**

1. Add the appropriate amount of medium to the vessels (1 ml/5 cm²) and allow the vessels to equilibrate in a 37°C, 5% CO₂, humidified incubator for at least 30 min.
2. Quickly thaw the cryovial in a 37°C water bath. Watch your cryovial closely; when the last sliver of ice melts remove it.
3. Resuspend the cells in the cryovial, dispense cells into the culture vessels set up earlier. Centrifugation is performed to remove cells from cryoprotectant cocktail.
4. Resuspend and plate the cells.
5. Change the growth medium the day after seeding and every other day thereafter.

**Subculturing:**

1. Subculture the cells when they are 60%-80% confluent.
2. Aspirate culture medium and wash cells 2x with 5 mL PBS
3. Cover the cells with 2 ml of Trypsin/EDTA solution. Examine the cell layer microscopically.
4. Allow the trypsinization to continue until approximately 90% of the cells are rounded up. (usually within 5 to 15 min)
5. Add 6 mL of complete medium and aspirate cells by gently pipetting
6. Add cells to a conical tube
7. Wash the flask with 5 mL medium and add the wash to the conical tube
8. Centrifuge the cells at 220 x g for 5 min.
9. Resuspend the cells in complete culture medium and add to a new flask
10. A subcultivation ratio of 1:5 is recommended

*Plate 40,000-50,000 cells/cm² and then grow them overnight or up to 24 hr

Ex. MTS/96 well plate 12,000-15,000 cells/well

Freezing Method
1. Subculture a confluent T75
2. Resuspend the cells in freeze medium
3. Split the cells 1:3 into cryovials
4. Freeze the cells at -80°C overnight then store them in liquid nitrogen

Culture of RLE-6TN Cells:
A rat alveolar type II epithelial cell line,
SV40 transfected (BSL2) (Non-tumerogenic)
Originator: KE Driscoll
Obtained from ATCC, catalog number CRL-2300
Lot 58005554 for NanoGO

Materials
Ham’s F12 medium with 1 mM L-glutamine (Gibco 11765-054)
L-glutamine (Sigma G-7513)
Bovine Pituitary Extract (BPE) (Sigma P-1167)
Insulin, bovine (Sigma I-0516)
Insulin-like growth factor (IGF-1) (Sigma I-8779)
Transferrin (Sigma T-1147)
Epidermal Growth Factor, mouse (EGF) (Sigma E-4127)
ATCC FBS (Do Not heat inactivate) (ATCC 30-2020)
Dulbecco’s PBS (ATCC 30-2200 or Gibco 14190-144)
Albumin from bovine serum (BSA) (Sigma A2058)
0.25% (w/v) Trypsin – 0.53 mM EDTA solution (ATCC 30-2101)
Penicillin-Streptomycin, 100x (Gibco 15140-122)

**Preparation and Storage of Materials**

<table>
<thead>
<tr>
<th></th>
<th>Weight (ug)</th>
<th>Solvent</th>
<th>Vol to Add (mL)</th>
<th>Final Conc (ug/mL)</th>
<th>Storage (°C)</th>
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</thead>
<tbody>
<tr>
<td>L-glutamine</td>
<td>already dissolved</td>
<td>cell culture grade H₂O</td>
<td>N/A</td>
<td>200*</td>
<td>-20</td>
</tr>
<tr>
<td>BPE</td>
<td>5000</td>
<td>sterile PBS</td>
<td>5</td>
<td>1000</td>
<td>-20</td>
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<tr>
<td>Insulin</td>
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<td>25 mM HEPES</td>
<td>N/A</td>
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<tr>
<td>IGF-1</td>
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<td>sterile filtered PBS with 0.1% BSA</td>
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<td>25</td>
<td>-20</td>
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<tr>
<td>Transferrin</td>
<td>100000</td>
<td>sterile filtered PBS with 0.1% BSA</td>
<td>10</td>
<td>10000</td>
<td>-20</td>
</tr>
<tr>
<td>EGF</td>
<td>100</td>
<td>sterile PBS</td>
<td>5</td>
<td>20</td>
<td>-20</td>
</tr>
</tbody>
</table>

*Concentration of L-glutamine expressed in mM

Note: All additives are cell culture grade and should not be sterile filtered

**Making Complete Culture Media**

Remove 12.9 mL of media from the stock bottle of Ham’s F12
Add all supplements to the 500 mL stock bottle according to the chart below
Mix the supplemented media (45 mL) with 10% FBS (5 mL) in a 50 mL conical tube
Use supplemented stock bottle within 3-4 weeks and FBS containing conical tubes within ~1 week
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamine*</td>
<td>200</td>
<td>1</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>BPE</td>
<td>1000</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Insulin</td>
<td>10000</td>
<td>5</td>
<td>0.25</td>
</tr>
<tr>
<td>IGF-1</td>
<td>25</td>
<td>0.0025</td>
<td>0.05</td>
</tr>
<tr>
<td>Transferrin</td>
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<td>1.25</td>
<td>0.0625</td>
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<tr>
<td>EGF</td>
<td>20</td>
<td>0.0025</td>
<td>0.0625</td>
</tr>
<tr>
<td>Pen-Strep**</td>
<td>10000</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total Vol Added</strong></td>
<td></td>
<td></td>
<td>12.925</td>
</tr>
</tbody>
</table>

*The final added concentration of L-glutamine is 1 mM, which makes the total concentration in the cell culture media 2 mM

**Concentration of Pen-Strep expressed in Units/mL

**Thawing Method**

Add 10 mL complete medium to a 15 mL conical tube, leave in hood at RT
Thaw cryovial in 37°C water bath
Pipette cells up and down to make sure they are suspended
Add cells drop wise to the conical tube of media
Centrifuge cells at 300 x g for 5 min
Resuspend cells in 15 mL complete media and add to a T75 flask

**Change media every 2-3 days**

**Grow 3-4 days allowing the cells to form a monolayer**

**Subculturing Protocol**

Aspirate culture medium and wash cells 2x with 5 mL PBS
Add 2-3 mL of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 min)
Add 6-8 mL of complete medium and aspirate cells by gently pipetting
Add cells to a conical tube
Wash the flask with 5mL medium and add the wash to the conical tube
Centrifuge the cells at 300 x g for 5 min
Resuspend the cells in complete culture medium and add to a new flask
A subcultivation ratio of 1:5 is recommended

*Plate 40,000-50,000 cells/cm² and then grow them overnight or up to 24 hr
Ex. MTS/96 well plate 12,000-15,000 cells/well

These cells have been shown to stay near diploid and karyotypically stable from passage 19-70 however a translocation did occur at passage 37 (ATCC). It is good practice to use cells at low passage numbers so plan to use the cells before passage 30-40.

**Freezing Method**
Make complete media with 15% DMSO
Add 0.5 mL to each labeled vial (3 vials per T75) and put on ice
Trypsinize and spin down cells
Resuspend in 1.5 mL (for 1 T75) of cold complete media (no DMSO)
Add 0.5 mL to each vial (final concentration 7.5% DMSO)
Quickly and gently vortex vials to mix
Freeze at -80°C overnight
Move to liquid nitrogen the next day

**MTS and LDH assays on BEAS-2B, RLE-6TN cells:**

**Seeding BEAS-2B, RLE-6TN into well plates**
The recommended density is 50,000 cells/cm² or 15,000 cells/well. 96 well plate (0.3 cm²/well).

**MTS cell viability assay**
Cellular viability is determined by the MTS assay, which measures the reduction of \{3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium} (MTS) to formazan by mitochondria in viable cells¹. After incubation with the indicated doses of NP for
various lengths of time at 37°C, formazan absorbance is measured at 490 nm. While a brief overview of the protocol is described here, the manufacturer's protocol is available online at http://www.promega.com/tbs/tb245/tb245.pdf

1. 1.5 X 10⁴ cells are plated onto 96 multi-well plates (Falcon Cat.# 353072) and grown for 24 hr until 70% confluent. Gently transfer culture plates to the incubator after plating so that cells do not clump in the center or periphery of the wells. NP are co-incubated with the cells for 6 and 16 or 24 hr (so, two time points) at concentrations of 1, 10, 25, 50, and 100 µg/ml. Controls are untreated cells that are harvested at both time points.

2. Thaw the CellTiter 96® AQueous One Solution Reagent from an aliquoted source immediately prior to use. Remove the original cell culture supernatant (containing the NP) from the wells and replace with 100 µl of fresh complete BEGM. Add 20 µl of the MTS reagent directly to each well.

3. The 96-well plates are incubated for 30 to 60 min at 37°C in a humidified, 5% CO₂ atmosphere.

4. Formazan absorbance is measured at 490 nm using a microtiter plate reader (e.g. Bio-Tek, Winooski, VT). The amount of colored product formed is proportional to the number of live cells in culture. If a treatment causes cell proliferation, viability will appear to increase.

5. An additional control, namely the MTS reagent mixed with NPs only, should be performed to ensure that there is no interference with the reagent.

6. Data analysis: Data are expressed as % cell viability. Mean absorbance of non-exposed cells (control cells) serves as the reference value for calculating 100% cellular viability. If x = mean value for control cells then the % cellular viability for samples (s) is calculated using the following equation: s = (mean value of sample/x)*100.

A.3. LDH assay (assessment of membrane integrity)

While a brief overview of the protocol is described here, the manufacturer's protocol is available online at http://www.promega.com/tbs/tb163/tb163.pdf http://www.promega.com/protcards/9fb054/fb054.pdf
1. The supernatant (50 µl) from the MTS assay is removed from the wells and transferred to a new 96-well plate.
2. Cells without treatment are used as the negative control. Cells treated with Triton-X100 are used as the positive control.
3. Add 50 µl of CytoTox-ONE™ Reagent to 100 µl of medium containing cells for the 96-well plate format. Incubate at room temperature for 30–45 min.
4. Add 50 µl of Stop Solution (per 100 µl of CytoTox-ONE™ Reagent added) to each well.
5. Record absorbance 490 nm.
PROTOCOL S2

Culture of THP-1 Cells

Description: Human monocyte from 1 year old male with acute monocytic leukemia exhibiting lymphoblast morphology and expressing Fc; complement (C3b). Antigen expression: HLA A2, A9, B5, DRw1, DRw2. THP-1 is a suspension cell culture that doubles in approximately 26 hours. The cells are phagocytic and lack surface and cytoplasmic immunoglobulin. Differentiation will be induced by a phorbol ester (phorbol 12-myristate 13-acetate) PMA (Sigma cat# P 8139) at 1 µg/ml. Inflammasome activation can be detected by monitoring IL-1β release, but it requires a low-level endotoxin co-exposure to induce pro-IL-1β, which is then cleaved by activated caspase 1. Biosafety Level 1.

Complete Growth Media: HEPES-buffered RPMI 1640 supplemented with L-glutamine (MediaTech Cellgro cat# 10-041-CV), 0.05 mM beta-mercaptoethanol and 10% heat-inactivated fetal bovine serum (PAA Laboratories cat # A15-204, lot # A20407-7003 if possible). No antibiotics. Filter media (0.2 µm) before use.

Freeze Media: Complete growth media with 5% (v/v) DMSO.

Cell Culture Methods: Obtain a vial from ATCC (cat# TIB-202, lot# 58636802)

• Quickly thaw the frozen contents.

• Once thawed, the cell suspension should be transferred to a 15 ml tube containing complete media (10 ml) and centrifuged at 300 x g for 5 min.

• The media supernatant should be discarded and the cell pellet should be resuspended in complete media (5 ml) and transferred to a T-25 cm² vented flask for culture in 37°C humidified environment with 5% CO₂. The optimal cell concentration is 2 – 4 x 10⁵ cells per ml.
• Avoid having the cell concentration exceed $1 \times 10^6$ cells per ml. Once the cell suspension reaches $8 \times 10^5$ cells per ml the cells can be subcultured in larger T-75 flasks. Change the media every 2 to 3 days depending on the cell density and growth rate.

• Keep track of the passages, as these cells can change over time. Do not use a cell lineage for longer than a month before you start a fresh line.

**Differentiation of THP-1 cells:**

• THP-1 cells can be differentiated into a macrophage-like cell by PMA (see above). The stock PMA should be 5000x (5 mg/ml in DMSO).

• Use 4 µl of the stock PMA solution per 20 ml (1 µg/ml) in the T-75 flask overnight to create an adherent cell that exhibits macrophage morphology. This process is optimal right after the media is changed or following fresh media exposure.

**Culturing the THP-1 with the nanoparticles:**

• After the cells are differentiated, they are washed with PBS 1x and then scraped off the plate into PBS (10 ml) by using a rubber cell scraper (Corning Cat# 3010).

• The resulting cell suspension is centrifuged at 300 x g for 5 min in 50 ml conical polypropylene tubes. The PBS supernatant is discarded and the cell pellet is resuspended in 1 ml of complete growth media (note: at this point conventional antibiotics can be used in the media if desired, since the cells are no longer proliferating).

• The cell number is then determined (we use a Z2 Coulter Counter, but a hemacytometer works also). The cells are suspended at $10^6$ cells per ml in complete growth media and exposed to nanoparticles in 1.5 ml microfuge tubes. Example: Depending on the number of cells, conditions, etc… typically place a 350 µl cell suspension into a 1.5 ml tube and
then pipette the particles in from a 5 mg/ml stock solution (ZnO is an exception at 1 mg/ml stock concentration). So 100 µg/ml would be 7 µl into the 350 µl, and 50 µg/ml would be 3.5 µl into the 350 µl, and 25 µg/ml would be 1.75 µl and 10 µg/ml would be 0.7 µl. Larger volumes can be used.

• This cell/particle suspension is then mixed by pipetting up and down while 100 µl is transferred in triplicate into 96-well tissue culture plates and placed in an incubator for 24 hr.

• Co-culture with a small amount of endotoxin is necessary for a proxy detection of inflammasome activation by IL-1β release. Lipopolysaccharide (LPS) can be obtained from Sigma (cat# L-4516 from E-coli 0127:B8 at 500,000 EU/ml).

• The stock solution should be 1000x at 10 µg/ml in sterile PBS. The final working concentration should be 10 ng/ml. This can be added to the complete culture media just prior to the particle exposure. The LPS has no direct effect or interaction effect with the particles on cell viability. Culturing the particle-exposed THP-1 cells without LPS usually results in little or no IL-1β release. Note: this can be done as an additional control, but it will create a very large experiment.

**Cytotoxicity Assays and IL-1β release:**

• At the end of the 24-hr culture period, transfer all of the culture media into a fresh 96-well plate with a multi-channel pipette and add back 120 µl complete culture media with the MTS reagent mixed in at a 1:6 ratio. Use Promega CellTiter 96™ Aqueous One solution kits (MTS reagent cat # 3580, 3581, 3582. This should be done in the following order.

• Thaw out the MTS reagent and prepare enough media/MTS reagent solution to do all of the wells plus 4 blank wells and some extra for pipetting error.
• Formula: \( 0.12 \times (\text{number of wells} + 4) = \text{ml of solution necessary for experiment} \).

• It is always prudent to make a little extra volume. If you need 12 ml for example (1 plate), this would be a combination of 10 ml complete media and 2 ml of MTS reagent.

• Once this is prepared, it can be placed in a sterile pipette basin under a sterile hood. Now the culture can be placed under the hood and the media transferred to another plate for cold (-20°C) storage and 50 µl isolated for the LDH assay.

• The media/MTS solution (120 µl/well) should be added to the original culture plate and this can be placed in the 37°C incubator for an additional 60 min. Do this quickly as the cells can be harmed by a protracted period of dryness.

• At the end of the 60 min period the plate contents should be mixed, and spun down at 2000 x g for 10 min.

• Take 100 µl of the media and transfer it into a fresh plate to be read at 490 nm. Avoid creating bubbles, because they distort the OD values. If bubbles are present, you can spin the plate at 1000 x g for 3 min to get rid of any bubbles before the read.

• The media/MTS solution blank (no cells) average should be subtracted from all samples, pseudo-replicates should be averaged and expressed as a percent relative to the no-particle control condition (100 %).

• The isolated 50 µl sample can be used for the LDH assay (use Promega CytoTox-ONE™ Homogeneous Membrane Integrity Assay cat # 7890, 7891). Perform the LDH assay immediately following the 24 hr culture as described in earlier NanoGo protocols in a manner consistent with the kit instructions. Fifty (50) µl of complete culture media can be used to determine the background levels of LDH from the serum supplement. This can then be subtracted out from all other sample values. The sample pseudo-replicates
should be averaged and expressed as a percent relative to the 100% total kill (note: a few no-particle control wells should be treated with 2 µl of the kit lysis solution cat # G182B 45 min prior to media collection). Again, be aware of mixing and the presence of bubbles before the final plate read.

- The remaining frozen 24-hr culture media can then be used for the IL-1β assessment (use R & D Systems Human IL-1β DuoSet cat# DY 201). From our experience, sample dilutions can range from 1:10 to 1:100 depending on the culture conditions (± LPS, ± particle for example). The standard curve for this assay is relatively small, so this sample dilution is critical for accurate results. Each lab has to determine the optimal dilution for their samples. In addition to the DuoSet, you will need ELISA plates (Nunc MaxiSorb cat # 439454) and color substrate (ThermoFisher 1-step Ultra TMB ELISA cat # 34028). A 1N sulfuric acid solution is used as a stop solution. For best results, use the R & D instruction sheet provided.
PROTOCOL S3

Protocols for BEAS-2B, RLE-6TN Cell Culture: No Change

Protocol for Biological assays: Modified MTS and LDH Protocol

A. Cell culture and co-incubation with nanoarticles

1. Seeding cells into multi-well plates
   - The recommended density is 50,000 cells/cm².
     6 well plate (9.6 cm²/well), 12 well plate (3.8 cm²/well), 24 well plate (2 cm²/well), 48 well plate (0.75 cm²/well), 96 well plate (0.3 cm²/well).

2. Validation of Culture Health
   - Prior to performing experiments on nanoparticles, each laboratory must demonstrate and characterize growth of healthy cells. Cell growth should be characterized and monitored for viability, growth and generation. Cell viability should remain >95% by Trypan blue exclusion.

3. Preparation of heat-inactivated FBS
   - Heat inactivation should be carried out in a pre-warmed water bath at 70°C for 5 min to fully inactivate LDH in serum. Thaw serum to room temperature and aliquot into 50 ml conical tubes, then place tubes into a 70°C water bath (the water bath should have enough water to allow the tubes sufficiently submerged).
   To fully inactivate LDH, keep the tubes inside the water bath for 8-10 min. To be more precise, you can use a tube of water as a blank and put a thermometer in it. When the blank reaches 70°C then start timing 5min.
4. Preparation of NP metal oxide suspensions

** Heat inactivated serum should only be used to prepare nanoparticle suspensions to decrease the background in LDH assays.**

- All the NP solutions are prepared fresh from stock solutions (e.g. 5 mg/ml). Stock solutions are made up from dry powder unless already in solution.

- An appropriate volume of WFI grade H$_2$O is added to the appropriate volume of particles. Dry powder NPs are weighed using a sensitive scale (e.g. Mettler Toledo Cat.# AL54) that can weigh mg quantities of material.

- The powder is then brought to a concentration equal to 5 mg/ml using cell WFI grade H$_2$O (Irvine Scientific, Cat.# 9309).

- For cell culture experiments this stock is vortexed (pulse for 5 sec) then sonicated for 15 min using Water Bath Sonicator (Branson, Model 2510, 100 W output power; 42 kHz frequency) immediately before diluting into complete cell culture medium (BEGM contains 2 mg/ml BSA, F12 containing 10% heat-inactivated fetal calf serum).

- Make the highest concentration suspension first. For instance, a 100 µg/ml exposure dose is made by adding 20 µl of the NP into 980 µl of complete culture medium.

- Sonicate the highest concentration suspension using a probe sonicator (Sonics and Materials Inc., Model vcx130, 130 W output power; 20 kHz frequency) at 40% amplitude for 15 sec followed by 15 sec break, then another 15 sec sonication.

- After sonication, make lower concentrations by making dilutions from the highest concentration. Vortex all suspensions again before cell treatment. The diluted NPs are inverted 3-4 times before each use to ensure that they stay well mixed.
5. Stability of NP suspensions

- The stability of the freshly prepared NP suspensions at 50 µg/mL is tested by light absorbance (UV-Vis) at 490 nm at 0, 3 and 24 hr after sonication and all measurements are done at room temperature.
- The stability of the freshly prepared NP suspensions can also be followed by dynamic light scattering (DLS) at 0, 3 and 24 hr after sonication (optional if the equipment is not available) and all measurements are done at room temperature.

6. Starting dosages:

- Starting dose range for ZnO is 4, 10, 25, 50 µg/ml.
- Starting dose range for TiO₂ P25, TiO₂ anatase, is 10, 25, 50, and 100 µg/ml.

**MTS and LDH assays on cells:**

- Seeding BEAS-2B, RLE-6TN into well plates
- The recommended density is 50,000 cells/cm² or 15,000 cells/well in 96 well plate (0.3 cm²/well). Culture volume is 100 µl.

**B. MTS cell viability assay**

MTS assay kit: Promega CellTiter 96® AQueous One Solution Cell Proliferation Assay PRODUCTS catalog number: G3580, G3581 AND G3582.

Cellular viability is determined by the MTS assay, which measures the reduction of \{3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium} (MTS) to formazan by mitochondria in viable cells. After incubation with the indicated doses of NP for 6 or 24 hr at 37°C, formazan absorbance is measured at 490 nm. The manufacturer's protocol is available online at http://www.promega.com/tbs/tb245/tb245.pdf.
To decrease particle interference with MTS assay, we made a change that is highlighted in the protocol that follows:

1. 1.5 X 10^4 cells are plated onto 96 multi-well plates (Falcon Cat.# 353072) and grown for 24 hr. Gently transfer culture plates to the incubator after plating so that cells do not clump in the center or periphery of the wells. NP prepared with media containing heat-inactivated serum or 2 mg/ml are co-incubated with the cells for 6 and 24 hr (two time points). Fresh culture media is put on the control cells. Controls are untreated cells that are harvested at both time points.

2. Thaw the CellTiter 96® AQueous One Solution Reagent from an aliquoted source immediately prior to use. Make a stock solution by combining cell culture medium (media without heat inactivation) (100 µl per well per number of wells) and MTS reagent (20 µl per well per number of wells). Remove the original cell culture supernatant (containing the NP) from the wells and replace with 120 µl of the stock solution containing MTS reagent directly to each well.

3. The 96-well plates are incubated for 60 min at 37°C in a humidified, 5% CO₂ atmosphere to allow fully color development.

4. After incubation, shake the plate for 5 sec in the plate reader or a shaker before formazan absorbance is measured at 490 nm by using a microtiter plate reader (e.g. Bio-Tek, Winooski, VT). Save the raw data for reference. After reading the plate, centrifuge the same plate at 2000 X g for 10 min to spin down the particles in the wells, then transfer 100 µl supernatant using a multichannel pipette (one row at a time) from each well to a new microtiter plate.

5. Spin the new plate at 1000 X g for 3 min to remove bubbles in the wells. Read the new plate at 490 nm, the data will be used for data analysis. The amount of colored product formed is proportional to the number of live cells in culture without nanoparticle interference.

6. Data analysis: Data are expressed as % cell viability. Mean absorbance of non-exposed cells (control cells) serves as the reference value for calculating 100% cellular viability. If x = mean value for control cells then the % cellular viability for samples (s) is calculated using the following equation: s = (mean value of sample/x)*100.
C. LDH assay (assessment of membrane integrity)

While a brief overview of the protocol is described here, the manufacturer's protocol is available online at http://www.promega.com/tbs/tb163/tb163.pdf

1. The supernatant after NP treatment in the original 96 well plate needs to be transferred to a new plate and centrifuged at 2000 x g for 10 min to spin down the cell debris and nanoparticles in the solution.

2. After centrifugation, the supernatant (50 µl) is removed from the wells and transferred to a new 96-well plate.

3. Cells without treatment are used as the negative control. Cells treated with 10 µl Lysis solution (provided in the kit) for 45 min are used as the positive control (this should be carried out 45 min before the end of the nanoparticle treatment for LDH assay are performed using the same plate).

4. Add 50 µl of CytoTox-ONE™ Reagent to each well and incubate at room temperature in dark for ~45 min.

5. Add 50 µl of Stop Solution to each well. Centrifuge at 1000 x g for 3 min to remove bubbles.

6. Record absorbance at 490 nm.
PROTOCOL S4

Protocol for using THP-1 cells in particle exposure model

General Description: Human monocytes from 1 year old male with acute monocytic leukemia exhibiting lymphoblast morphology and expressing Fc; complement (C3b). Antigen expression: HLA A2, A9, B5, DRw1, DRw2. THP-1 is a suspension cell culture that doubles in approximately 26 hours. The cells are phagocytic and lack surface and cytoplasmic immunoglobulin. Differentiation will be induced by 1, 25-Dihydroxy-Vitamin D₃ at 150 nM. Inflammmasome activation can be detected by monitoring IL-1β release, but it requires a low-level endotoxin co-exposure to induce pro-IL-1β, which is then cleaved by activated caspase 1 as a result of inflammasome NLRP3 assembly. Biosafety Level 1.

Materials necessary for experiment:

Culture items:
- THP-1 cell line - ATCC (cat# TIB-202)
- Complete Growth Media: HEPES-buffered RPMI 1640 supplemented with L-glutamine (MediaTech Cellgro cat# 10-041-CV or similar), 0.05 mM beta-mercaptoethanol and 10% heat-inactivated fetal bovine serum (PAA Laboratories cat # A15-204, lot # A20407-7003 or similar). Filter media (0.2 µm) before use.
- Freeze Media: Complete growth media with 5% (v/v) DMSO.

Reagents to be used:
- 1, 25-Dihydroxy-Vitamin D₃ (50 µg, EMD cat# 679101).
- Phorbol, 12-myristate, 13-acetate (PMA in DMSO) (2 x10 µl at 1 mg/ml (1.62 mM) Sigma cat# P8139).
- Lipopolysaccharide (LPS) (1 mg, Sigma cat# L-4516 from E-coli 0127).
- LDH assay (use Promega CytoTox-96™ Homogeneous Membrane Integrity Assay cat# G1780).
- MTS assay (use Promega CellTiter-96™ One Solution cat# G3580).
• Human IL-1β ELISA (use R & D Systems Human IL-1β DuoSet™ cat# DY 201). In addition to the DuoSet, you will need ELISA plates (Nunc MaxiSorp cat # 439454) and color substrate (ThermoFisher 1-step Ultra TMB ELISA cat # 34028). A 2N sulfuric acid solution is used as a stop solution.

**Cell Culture Methods:**

• Quickly thaw the frozen contents.

• Once thawed, the cell suspension should be transferred to a 15 ml tube containing complete media (10 ml) and centrifuged at 300 x g for 5 min.

• The media supernatant should be discarded and the cell pellet should be resuspended in complete media (1 - 5 ml) and transferred to a T-75 cm² vented flask for culture (20 ml total volume) in 37°C humidified environment with 5% CO₂. The optimal cell concentration is 2 – 4 x 10⁵ cells per ml.

• Avoid having the cell concentration exceed 1 x 10⁶ cells per ml. Change the media every 2 to 3 days depending on the cell density and growth rate.

• Keep track of the passages, as these cells can change over time. Do not use a cell lineage for longer than a month before you start a fresh line.

**Differentiation of THP-1 cells:**

• THP-1 cells can be differentiated into a macrophage-like cell by Vit D₃. The stock Vit D₃ should be 100 µM (in 100% EtOH).

• Use 30 µl of the stock Vit D₃ solution per 20 ml (150 nM) in the T-75 flask overnight to create a semi-adherent cell that exhibits macrophage morphology. The cells will become completely adherent during the particle exposure phase of the experiment. **Note:** The
experiment requires approximately $10 \times 10^6$ cells so prepare enough flasks to have more than this amount to be safe.

**Culturing the THP-1 with the nanoparticles:**

- After the cells are differentiated, they will appear to be semi-adherent. Use a rubber cell scraper (Corning Cat# 3010) to dislodge the sticking cells and place the resulting cell suspension in a 50 ml polypropylene centrifuge tube.

- The cell suspension is then centrifuged at 300 x $g$ for 5 min. The media supernatant is discarded and the cell pellet is resuspended in 5 ml of complete growth media.

- The cell number is then determined (such as a $Z_2$ Coulter Counter, but a hemacytometer works also).

- The cells are suspended at $10^6$ cells per ml in complete growth media.

- Co-culture with a small amount of endotoxin (LPS) is necessary for a proxy detection of NLRP3 inflammasome activation by IL-1β release. The stock solution of LPS should be 1000x at 10 µg/ml in sterile PBS. The final working concentration should be 10 ng/ml (1 µl/ml). This can be added to the complete culture media just prior to the cell/particle exposure. The LPS has no direct effect or interaction effect with the particles on cell viability. A small amount of PMA (10 nM) is added at this time also activate the macrophage-like cells. Use a 10 µM stock solution in 100% DMSO. This will be a 1:1000 dilution.

- Expose cells to nanoparticles in 1.5 ml microfuge tubes. **Example:** Depending on the number of cells, conditions, etc… typically place a 350 µl cell suspension into a 1.5 ml tube and then pipette the particles in from a 5 mg/ml stock solution. 100 µg/ml would be
7 µl into the 350 µl, and 50 µg/ml would be 3.5 µl into the 350 µl, and 25 µg/ml would be 1.75 µl and 10 µg/ml would be 0.7 µl. Larger volumes can be used.

- This cell/particle suspension is then mixed by pipetting up and down as 100 µl is transferred in triplicate into 96-well tissue culture plates and placed in an incubator for 24 hr at 37°C.

Assays (MTS and LDH cytotoxicity) and IL-1β release:

- At the end of the 24-hr culture period add 10 µl lysis buffer (cat # G182A) to the 3-“100% Kill” wells and return to incubator for 40 minutes.

- Thaw out the MTS reagent (CellTiter 96 One Solution) and prepare enough media/MTS reagent solution to do all of the wells plus 3 blank wells and some extra for pipetting error.

- Formula: 0.12 x (number of wells + 4) = ml of solution necessary for experiment. It is always prudent to make a little extra volume. If you need 12 ml for example (1 plate), this would be a combination of 10 ml complete media and 2 ml of MTS reagent.

- After 40 min in the incubator, spin the plate at 1000 x g for 3 min @ RT.

- 50 µl of media per well should be transferred to a fresh plate for the LDH assay. Care should be taken not to disrupt the cells while pipetting or to introduce bubbles into the new plate.

- In the fresh plate, add 50 µl of LDH assay substrate (part# G179A) (made fresh with 11 ml of substrate buffer (part# G180A)) to each well of LDH assay plate. Let plate develop for approximately 10 min @ RT (exact times may vary – darkest wells should not be > 2.0 OD value).
• While the LDH assay is developing, transfer the remaining media from the original cell culture plate into a fresh plate. Take care to not disrupt the cells while pipetting. Place this plate in cold (-20°C) storage for later IL-1β assay. **Note:** at this point all loose material is gone from the plates.

• The media/MTS solution (120 µl/well) should be added to the original culture plate and this can be placed in the 37°C incubator for an additional 40 - 60 min depending on development speed. Do this quickly as the cells can be harmed by a protracted period of dryness (exact timings may vary – darkest wells should not be > 2.0 OD value).

• Add 50 µl of stop solution (part# G183A) to each well of LDH assay plate and read at 490 nm. The “media only” wells can be subtracted out as background. Some plate-reader allow to designate these as “blanks”, and they are automatically subtracted out from all observations. The sample pseudo-replicates should be averaged and expressed as a percent relative to the “100% total kill” average.

• At the end of the 40 to 60 min period, transfer 100 µl of the media/MTS solution into a fresh plate. The color development in the wells will not be uniform, so it is necessary to pipette up and down once (gently) to mix the color in the media before transfer. Avoid creating bubbles, because they will distort the OD values. If bubbles are present in any wells, spin the plate at 1000 x g for 3 min to get rid of any bubbles before the read.

• Read the plate at 490 nm. The media/MTS solution blank (no cells) average should be subtracted from all samples (see above). Pseudo-replicates should be averaged and expressed as a percent relative to the no-particle control condition (100 %).

• The frozen 24-hr culture media can then be used for the IL-1β assessment. From experience, sample dilutions can range from 1:50 to 1:300 depending on the culture conditions (± LPS, ± particle for example). A 1:100 dilution is a starting place. The standard curve for this assay is relatively small window, so this sample dilution is critical
for accurate results. Each lab has to determine the optimal dilution for their samples. For best results, use the R & D instruction sheet provided.
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**Table S3:** Statistical analyses of BEAS-2B toxicity data. Data analysis consisted of a 2-way ANOVA followed by Tukey’s correction for multiple comparisons of means. Significance indicated at P (probability of type I error) < 0.05, 0.01, or 0.001 compared to 0 µg/ml concentration and other particles at the same concentration. Red font indicates concentration in µg/ml. *ns* indicates not significant.
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**Table S4:** Statistical analyses of RLE-6TN toxicity data. Data analysis consisted of a 2-way ANOVA followed by Tukey’s correction for multiple comparisons of means. Significance indicated at $P$ (probability of type I error) < 0.05, 0.01, or 0.001 compared to 0 µg/ml concentration and other particles at the same concentration. Red font indicates concentration in µg/ml. ns indicates not significant.
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**Table S5**: Statistical analyses of THP-1 toxicity and IL-1β data. Data analysis consisted of a 2-way ANOVA followed by Tukey’s correction for multiple comparisons of means. Significance indicated at $P$ (probability of type I error) < 0.05, 0.01, or 0.001 compared to 0 µg/ml concentration and other particles at the same concentration. Red font indicates concentration in µg/ml. ns indicates not significant.