2. Methods

- PROTEIN DETERMINATION & WESTERN BLOT

Protein assay of 9-10-2010 lung homogenates mg protein/mL Bio-Rad Assay

Amount of protein from six lung homogenate samples in 10 µL, added to gel

<table>
<thead>
<tr>
<th>Protein Amount (µg)</th>
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<tr>
<td>17.8 µg</td>
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<td>17.9 µg</td>
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<td>20.9 µg</td>
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<tr>
<td>14.6 µg</td>
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<tr>
<td>15.4 µg</td>
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<td>20.0 µg</td>
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1. Use samples under 50 micrograms based on protein assay.
2. Dilute 50/50 with Lamelli buffer, heat 95 C 3 min and run on 12% Bio-Rad precast gel.
3. Blot to nitrocellulose in cold for 2 hr.
4. Incubate overnight with anti-RAGE, anti TLR 4 or 9.
5. Dilute 50/50 with buffer, heat 95 C 3 min and run on 12% Bio-Rad precast gel.
6. Use samples under 50 micrograms based on protein assay.
7. With scans into "Image J" software, Intensity of each receptor band divided by intensity of B actin band.

3. Results

**Cell bound and Soluble HMGB1 receptors released into lung following nanoparticle exposure**

- **0.5 hr**
  - TLR4 ligand
  - TLR4 4 no effect
  - TLR9 no effect
  - RAGE no effect

- **3 hr**
  - TLR4 small block of ligand
  - TLR9 small block of ligand
  - RAGE binds ligand

- **24 hr**
  - TLR4 ligand block-soluble high
  - TLR9 small block of ligand
  - RAGE binds ligand

- **4 weeks**
  - TLR4 ligand block-soluble
  - TLR9 small block of ligand
  - RAGE binds ligand

4. Discussion & Summary

- **0.5 hr**: data suggest the high HMGB1 at 0.5 hr will have little cytokine effect since TLR4 is the major receptor present and is soluble, blocking soluble HMGB1.
- **3 hr**: data show RAGE has high potential to bind HMGB1 at 3 hr. Soluble TLR-9 is high and could block the low HMGB-1 at 3 hr.
- **24 hr**: data suggest the highest cytokine stimulation occurs at 24 hr, a high HMGB1, no soluble RAGE, but cell-associated RAGE receptor. Potential blocking from soluble TLR-4.
- **4 weeks**: High HMGB1 good cell-bound RAGE, low soluble (blocking) RAGE and some blocking from both TLR9 and 4.

6. Limitations

- Paper does not include the cytokines stimulated by the cell bound receptors and HMGB1.
- We limited the receptors studied to TLR4, TLR9 and RAGE, though HMGB1 has other known receptors as well.

7. References