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Assay to Evaluate BAL Fluid Regulation of Fibroblast α-SMA Expression

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[Abstract] Because transforming growth factor-β (TGF-β1) induces differentiation of fibroblasts to myofibroblasts, we developed a protocol to evaluate alveolar macrophage-derived TGF-β1 regulation of lung fibroblast differentiation (Larson-Casey *et al*., 2016). The protocol evaluates the ability of mouse bronchoalveolar lavage (BAL) fluid to alter fibroblast differentiation. Fibroblast differentiation was measured by the expression of α-smooth muscle actin (α-SMA).

[Background] Alveolar macrophages play an integral role in pulmonary fibrosis development by increasing the expression of TGF-β1 (He *et al*., 2011). Our prior data demonstrate that alveolar macrophages are a critical source of TGF-β1 as mice harboring a conditional deletion of TGF-β1 in macrophages were protected from pulmonary fibrosis (Larson-Casey *et al*., 2016). The expression of α-SMA is a defining feature of myofibroblasts, and TGF-β1 is a well-characterized pro-fibrotic mediator that induces transformation of fibroblasts to myofibroblasts both *in vitro* (Desmoulière *et al*., 1993) and *in vivo* (Sime *et al*., 1997). Prior studies exposed fibroblasts to recombinant TGF-β1 to show its effect on differentiation and function (Horowitz *et al*., 2007). Here we have developed a protocol for determining the ability of mouse BAL fluid to alter the differentiation of human lung fibroblasts to myofibroblasts, the cells that produce extracellular matrix proteins.

Materials and Reagents

1. 6-well cell culture plates (Corning, Costar®, catalog number: 3516)
2. Normal human fibroblasts (IMR-90) (ATCC, catalog number: CCL-186)
3. DMEM
4. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 26140095)
5. Penicillin-streptomycin (10,000 U/ml, 10,000 µg/ml) (Thermo Fisher Scientific, Gibco™, catalog number: 15140122)
6. Amphotericin B (Thermo Fisher Scientific, Gibco™, catalog number: 15290018)
7. RPMI 1640 medium, no phenol red (Thermo Fisher Scientific, Gibco™, catalog number: 11835030)
8. DPBS (Thermo Fisher Scientific, Gibco™, catalog number: 14190144)
9. NP-40

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10. Sodium chloride (NaCl)
11. Protease inhibitor tablets (Sigma-Aldrich, catalog number: 11836170001)
12. Phosphatase inhibitor (EMD Millipore, catalog number: 524625)
13. α-SMA antibody (American Research Products, catalog number: 03-61001)
14. β-actin antibody (Sigma-Aldrich, catalog number: A5441)
15. Tween 20
16. Fibroblast culture media (see Recipes)
17. Lysis buffer (see Recipes)

Equipment

1. Cell culture incubator, 37 °C, 5% CO_2: 95% air atmosphere (Thermo Fisher Scientific, Forma™, model: Direct Heat CO_2 Incubator)

Procedure

1. Seed fibroblasts at a density of 1 x 10^6 per well of a 6-well cell culture plate in a total volume of 1 ml. Allow cells to adhere to cell culture plate (2-6 h).
2. Harvest bronchoalveolar lavage (BAL) fluid from mice (Han and Ziegler, 2013). Mice can be treated with bleomycin (1.75 U/kg) or saline as a negative control. Determine the protein concentration of bronchoalveolar lavage (BAL) fluid. Using RPMI 1640 media, normalize all BAL samples to the same protein concentration in a total volume of 1 ml.
   Notes:
   a. *Typically, 0.5-1 ml of BAL fluid is used and RPMI is used to normalize the protein concentration to a final total volume of 1 ml.*
   b. *Alternatively, this assay can be performed in vitro using conditioned media from macrophage cell lines or bone marrow derived macrophages. Harvest the conditioned media from macrophages post-transfection or post-treatment, using 1 million cells per 1 ml of media.*
3. Remove media from fibroblasts and rinse with 1.5 ml room temperature 1x PBS, add normalized BAL fluid to fibroblasts.
   a. Incubate fibroblasts with BAL fluid for 24 h in a cell culture incubator, 37 °C, 5% CO_2: 95% air atmosphere.
   b. Harvest fibroblasts by lysing cells in lysis buffer (see Recipes).
4. Determine α-SMA expression by immunoblot analysis. The expression of α-SMA protein was determined by Western blotting using 20 µg of total cellular protein. After blocking in 5% milk, the membranes were probed with mouse α-SMA primary antibody using a 1:3,000 dilution in Tris buffered saline containing 0.1% Tween 20, the membranes were incubated with the anti-mouse secondary antibody (1:2,000) in antibody dilution buffer for 1 h.

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Data analysis

Results can be shown as a representative immunoblot (Figure 1); also see Larson-Casey *et al*. (2016).

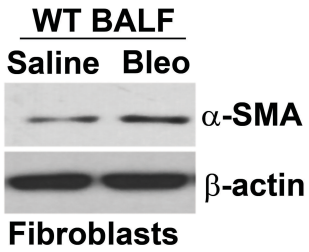

Figure 1. Immunoblot analysis of α-SMA in IMR-90 fibroblasts cultured in BAL fluid (BALF) from saline or bleomycin (Bleo) exposed WT mice

Recipes

1. Fibroblast culture media
   DMEM
   10% heat-inactivated FBS
   100 U/ml, 100 µg/ml penicillin-streptomycin
   1.25 µg/ml amphotericin B (fungizone)
2. Lysis buffer
   1% NP-40
   0.15 M NaCl
   0.05 M Tris pH 7.4
   1 protease tablet
   Phosphatase inhibitor diluted 1:100

Acknowledgments

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