Dysregulated S1P Signalling in a Mouse Model of Cystic Fibrosis-like Lung Disease Produced by β-ENaC Overexpression

Matthew G Macowan[1,2], Hai B Tran[1,2], Kate L Matthews[2], Melanie Ralph[1,2], Martin Donnelley[2-4], David Parsons[2-4], Sandra Hodge[1,2]

[1]Department of Thoracic Medicine, Royal Adelaide Hospital; [2]Adelaide Medical School, University of Adelaide; [3]Robinson Research Institute, University of Adelaide; and [4]Women's and Children's Hospital, Adelaide

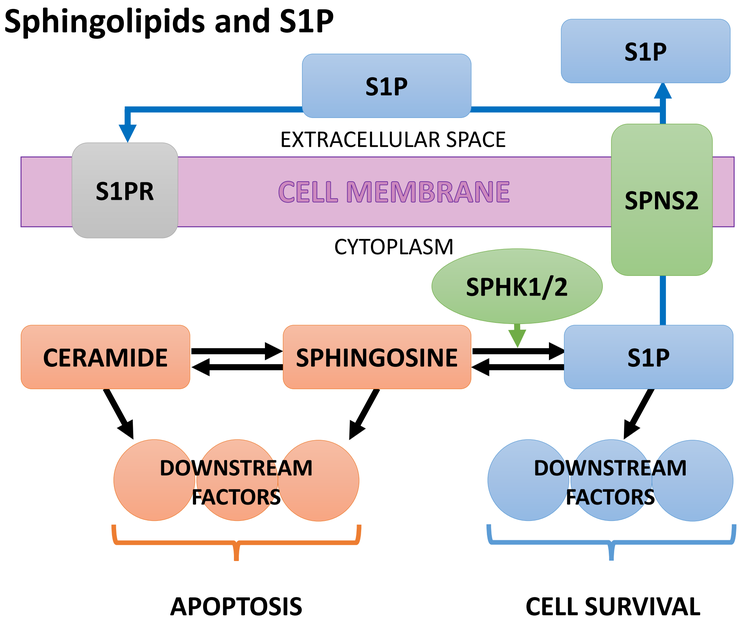
INTRODUCTION

- **Cystic fibrosis (CF) is a life-threatening, multi-organ disease.** The lung is the primary target, and results in the largest morbidity and mortality.
- Epithelial dehydration via defective CFTR activity results in the hallmark **mucus obstruction** and chronic, destructive **pro-inflammatory conditions** in the lower airways.
- Prior studies suggest that mechanisms of **CF may include dysregulated sphingolipid signalling**[1,2].

Inflammation

- CF lungs exhibit elevated levels of IL-1β, TNFα, IL-6, and IL-8.
- **IL-1β is controlled via activation of inflammasomes**; cytosolic multiprotein complexes aggregated around a sentinel protein, e.g. NLRP3.
- **NLRP3 inflammasome strongly implicated in CF**[3], but unclear whether this is due to CF or downstream stimuli such as pathogen presence, e.g. *P. aeruginosa*.

Sphingolipids and S1P



- Sphingosine-1-phosphate (**S1P**) is a small, bioactive lipid that regulates many cellular processes and is important in airway diseases[4].
- **S1P is generated in cells from sphingosine by SPHK1 and SPHK2**.
- It acts on intracellular and membrane-bound receptors (S1PRs), and can be **exported from the cell by SPNS2** (Spinster homologue 2) where is can act via autocrine and paracrine signalling.
- **S1P opposes sphingosine and ceramide** from which it is derived → this balance is known as the sphingosine rheostat.
- Ceramide has been identified as a potential cause of **cell death**, **inflammation**, and **infection susceptibility** in CF patients and *CFTR* mutant mice.

We therefore assessed a CF-like mouse model (βENaC) for its efficacy for further investigations into potential S1P dysregulation in CF disease.

βENAC MODEL

- Transgenic mouse model over-expressing the β-subunit of the epithelial sodium channel (ENaC).
  - Mimics the Na^+ ion transport abnormalities observed in CF airways.
  - Increased Na^+ absorption → depletion of airway surface liquid and deficient mucus clearance.
- Exhibit CF-like lung disease features[5]:
  - Mucus hypersecretion.
  - Mucus obstruction in the conducting airways.
  - Mucociliary clearance impairment.
  - Goblet cell metaplasia.
  - Airway inflammation.
  - Poor bacterial clearance following intra-tracheal pathogen challenge with *H. influenzae* and *P. aeruginosa*.
- Is reported to be a relevant model for investigating certain elements of CF lung disease[5].

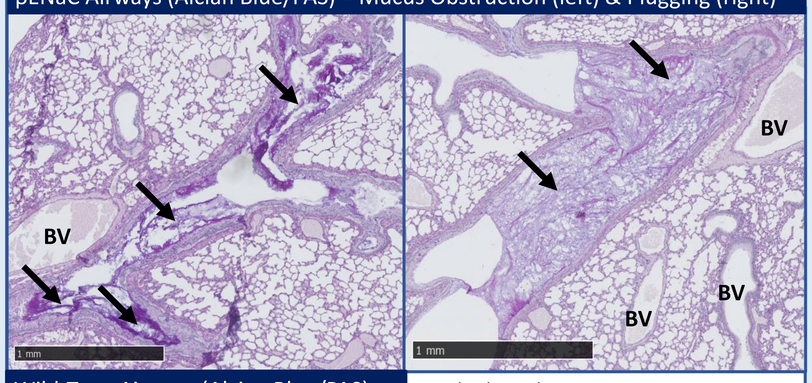
METHODS

- Archived mouse lung tissue sections from βENaC mice and wild-type littermates were retrieved. Section availability varied by individual mouse ID, sometimes altering n values.
- Tissue sections were stained for key proteins and observed by immunofluorescence via confocal microscopy at 60x objective magnification. Mucus obstruction and plugs were identified by Alcian Blue/PAS staining and microscopy.

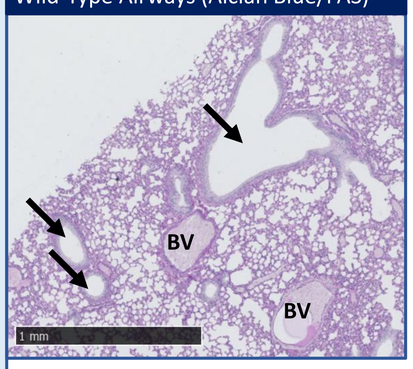
RESULTS

Confirmation of Mucus Obstruction in Conducting Airways

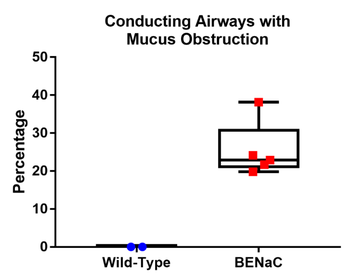
βENaC Airways (Alcian Blue/PAS) – Mucus Obstruction (left) & Plugging (right)



Wild-Type Airways (Alcian Blue/PAS)



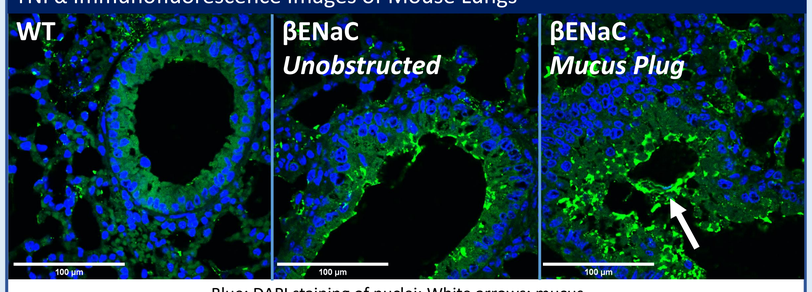
BV: Blood Vessel
Black arrows: conducting airways



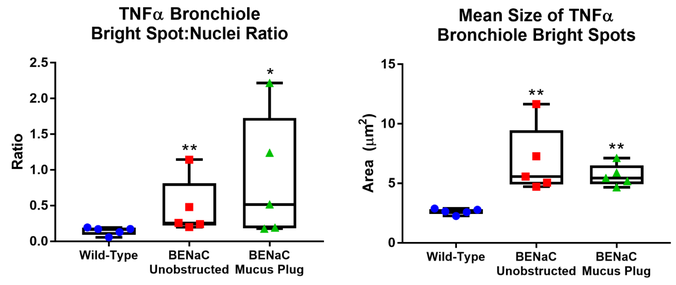
As previously shown, **βENaC mice develop certain hallmark characteristics of CF** such as mucus accumulation and obstruction of the airways. In the mice examined, **approximately 25.3% of the conducting airways showed some degree of mucus accumulation**.

Inflammatory Airway Environment with βENaC Over-Expression

TNFα Immunofluorescence Images of Mouse Lungs



Blue: DAPI staining of nuclei; White arrows: mucus



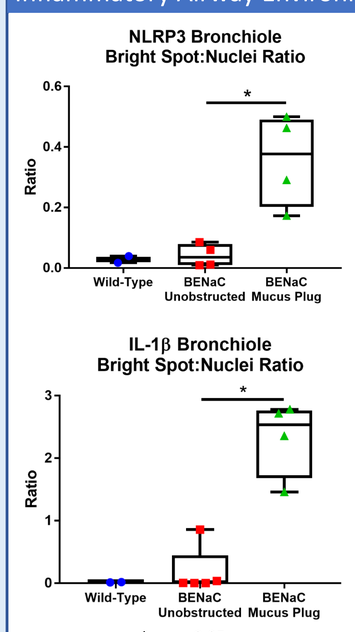
* = p < 0.05; ** = p < 0.01

The **inflammatory cytokine TNFα was overexpressed in conducting airways of βENaC mice**, irrespective of mucus obstruction. The number of TNFα immunofluorescence bright spots and their average size were similar in these mice, while control mice bronchioles showed only dull staining.

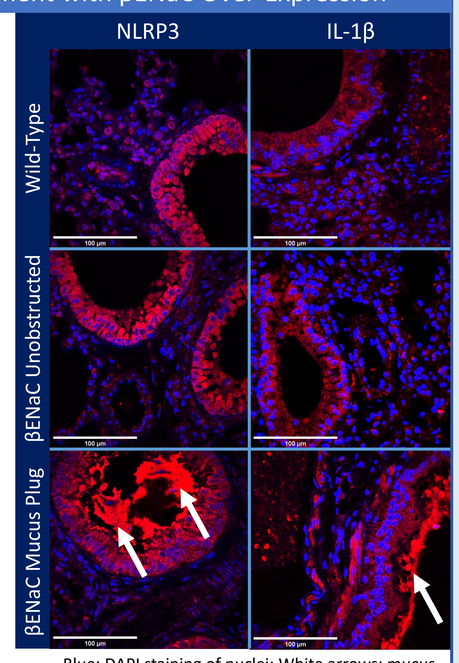
REFERENCES

1. Teichgräber V et al., 2008. 'Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis,' *Nat Med* **14**(4): 382-391.
2. Ebenezer DL et al., 2016. 'Targeting sphingosine-1-phosphate signalling in lung diseases,' *Pharmacol Ther* **168**: 143-157.
3. Iannitti RG et al., 2016. 'IL-1 receptor antagonist ameliorates inflammasome-dependent inflammation in murine and human cystic fibrosis.' *Nat Commun* **7**:10791.
4. Maceyka M et al., 2012. 'Sphingosine-1-phosphate signalling and its role in disease,' *Trends Cell Biol*, **22**(1): 50-60.
5. Mall M et al., 2004. 'Increased airway epithelial Na^+ absorption produces cystic fibrosis-like lung disease in mice,' *Nat Med* **10**:487-493.
6. Baroja-Mazo A, 2014. 'The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response,' *Nat Immunol* **15**: 738-748.

Inflammatory Airway Environment with βENaC Over-Expression



* = p < 0.05

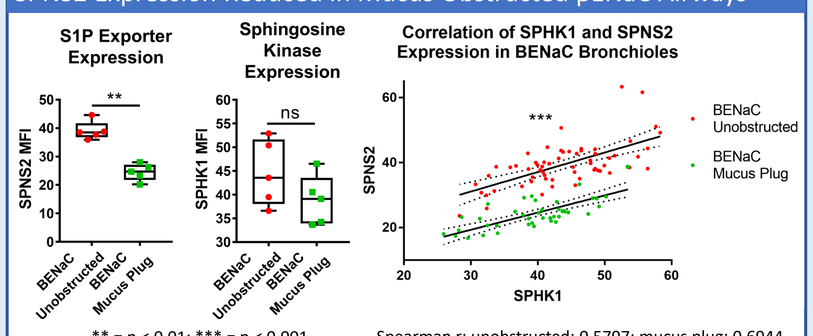


Blue: DAPI staining of nuclei; White arrows: mucus

NLRP3 significantly increased in βENaC mice with mucus presence with some extracellular localisation in bronchiole lumen; NLRP3 reportedly released by cells where it acts an extracellular oligomeric complex to amplify the inflammatory response[6]. **Downstream IL-1β also showed significantly increased levels**.

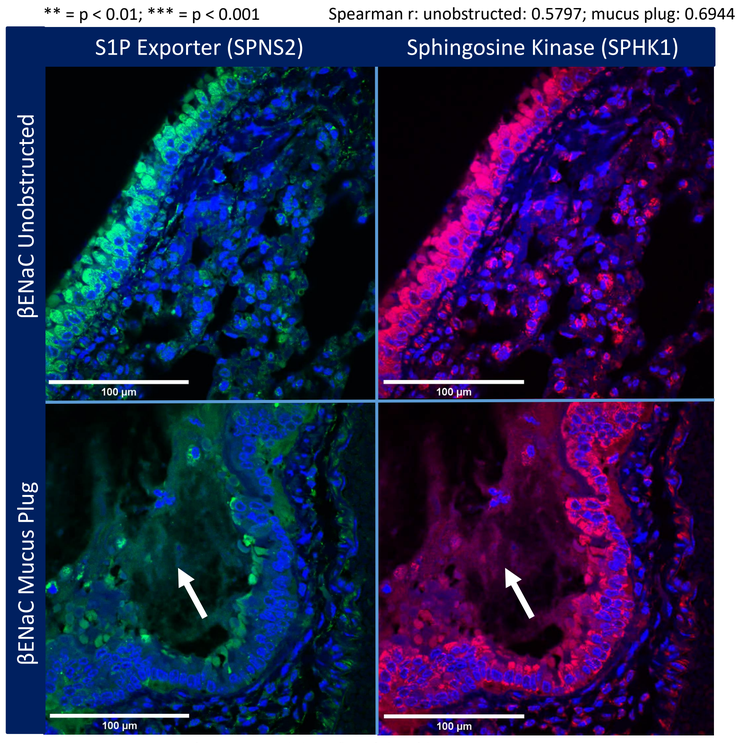
Suggests while βENaC mice demonstrate genotypic increases in inflammation, inflammasome-mediated inflammation may relate to airway mucus presence.

SPNS2 Expression Reduced in Mucus Obstructed βENaC Airways



** = p < 0.01; *** = p < 0.001

Spearman r: unobstructed: 0.5797; mucus plug: 0.6944



Blue: DAPI staining of nuclei; White arrows: mucus

Mucus presence significantly decreases expression of SPNS2 in βENaC mice in addition to a **trend for decreased SPHK1 expression**; the **expression levels of these proteins are significantly correlated.**

CONCLUSIONS

- The βENaC model successfully developed some CF-like characteristics, particularly in regard to mucus and inflammation.
- Mucus-related downregulation of SPHK1 and SPNS2 may point to dysregulation of the S1P pathway.
  - Potential intracellular build-up of S1P (need to test S1P phosphatase levels (SGPP1); reverses action of SPHK1).
- As this model does not involve dysfunctional CFTR, we aim to compare it with a *CFTR*$^{-/-}$ KO rat model to determine its efficacy for S1P signalling experiments.