I. ANIMAL EXPERIMENTS

In the first reporting period, a BOS mouse model was set up in which subcutaneous trachea transplantation was carried out between C57BL/6 and Balb/c mice (n=140) (Figure 1).

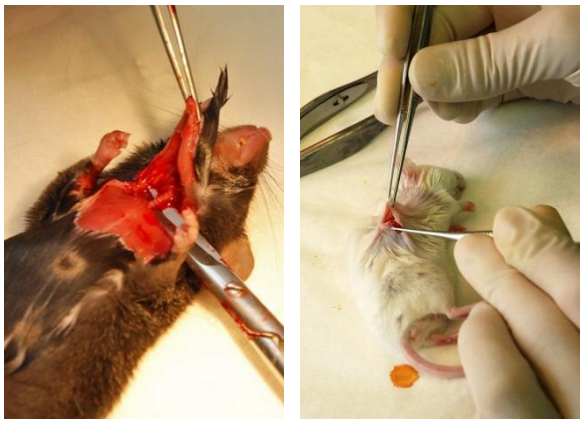

Figure 1: Subcutaneous trachea transplantation between C57BL/6 and Balb/c mice

The day after the transplantation, intraperitoneal treatment of adrenomedullin (ADM), cyclosporine-A (CyA), adrenomedullin with cyclosporine A (ADM+CyA) and saline (control group, K) was begun for 5 weeks, twice daily. Sampling was carried out weekly, including blood (sera) for protein analysis and tissue (trachea graft) for immunohistochemistry and qRT-PCR. So, altogether, there were 20 groups (5 weeks*4 treatment). After a total RNA extraction from the trachea grafts, gene expression analysis of IL-6, TGF-β and INF-γ by real time PCR was carried out using β-actin as reference gene, followed by reverse transcription. The results of the gene expression are shown on Figure 2. Statistical differences between the groups were tested by Mann-Whitney U test or by Kruskal–Wallis ANOVA and median test. Differences were considered statistically significant when $p<0.05$. All statistical analyses were performed using the STATISTICA software. On the first week an IL-6 expression decrease was observed between the treated and the control groups. The difference between the control and CyA+ADM treated group was significant ($p<0.001$), but the difference between the CyA+ADM and CyA treated groups was not significant, thus the effect of the ADM was not detectable. The expression of TGF- β showed higher levels in the control group than in the treated groups, but the differences were not significant.

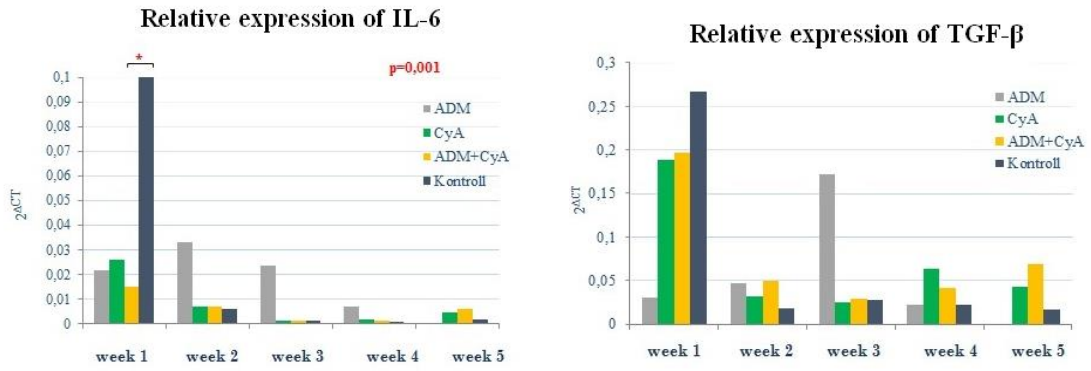

Figure 2: The results of the gene expression of the IL-6 and the TGF-β.

Contrary to our expectations, the first set of experiments showed the BOS model was not suitable for the detection of the effect of adrenomedullin. To confirm these results, LUMINEX assay of TGF-β, TNF-α and IL-2 expression was performed from blood serum samples of mice, in collaboration with the University of Pécs, Department of Pharmacology and Pharmacotherapy. Results showed a huge range in expression levels and the difference between the control group (treated with saline) and the adrenomedullin treated group (treated with adrenomedullin intraperitoneally twice a day (108 ng/12h) was not significant. Figure 3 shows the results of the LUMINEX assay.

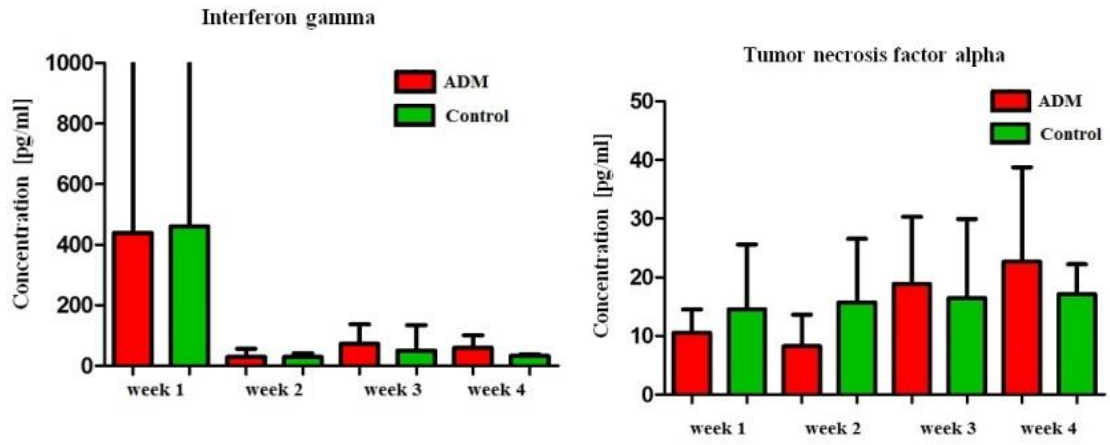

Figure 3: The results of the LUMINEX assay of IFN-γ and TNF-α.

Although the BOS model is suitable for examining the progression of BOS (luminal obliterations), it still has some limitations. The half of the trachea graft was formalin fixed and paraffin embedded, and after the hematoxylin-eosin (HE) staining, semi-quantitative analysis

of FFPE tissue samples by expert pathologists was also carried out. The HE staining showed difference between the group treated with ADM+CyA and the control group (treated with saline). Figure 4 shows some representative slides from each group.

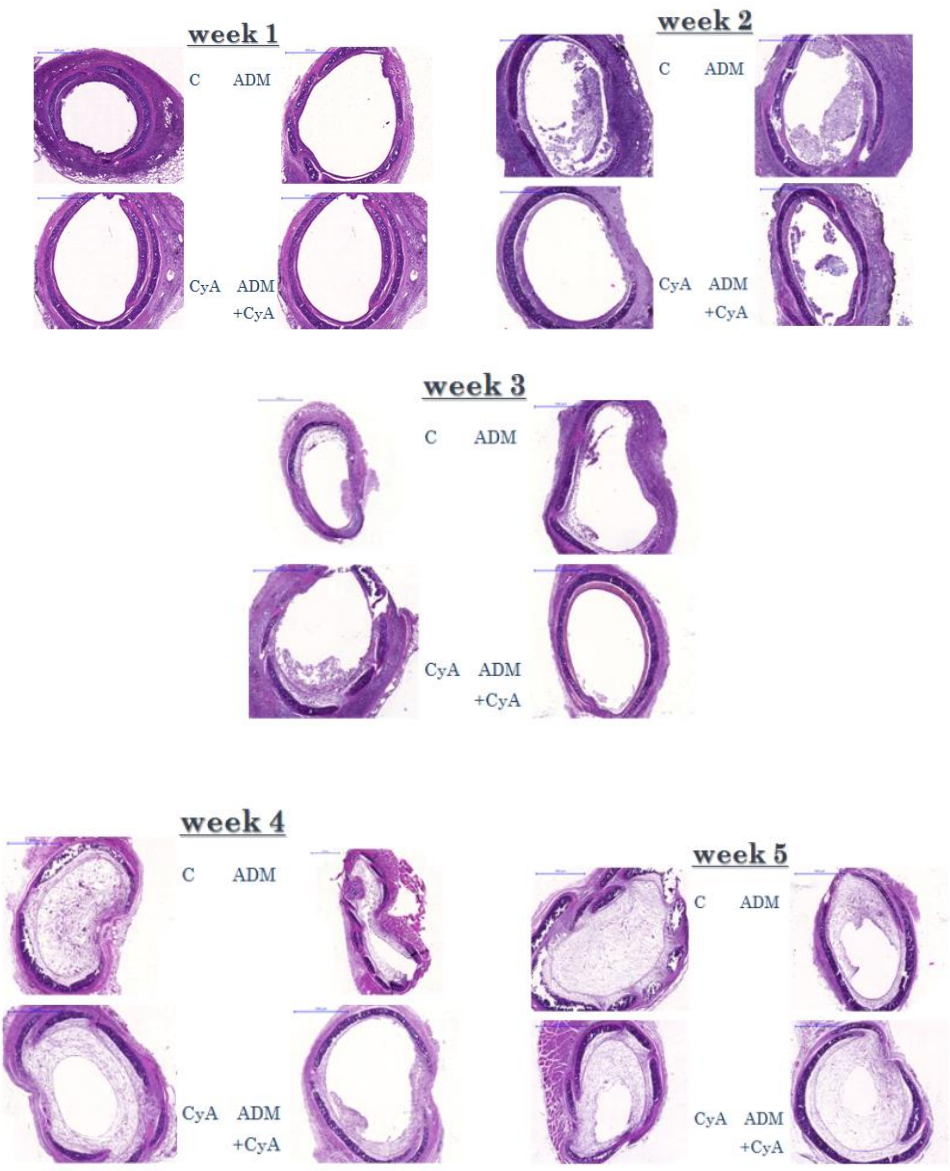

Figure 4: Representative slides of the analyzed tissue grafts. C: Control (saline treated), ADM: adrenomdullin treated, CyA: Cyclosporine treated, ADM+CyA: adrenomedullin and cyclosporine treated groups.

To evaluate the obliteration and the inflammation, a score scale was used. Results of this semi-quantitative analysis were shown on Figure 5 and 6.

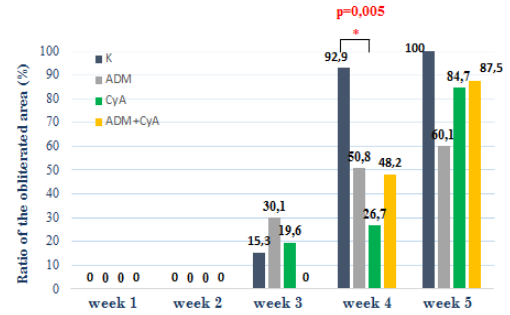

Figure 5: Obliteration scores of the groups

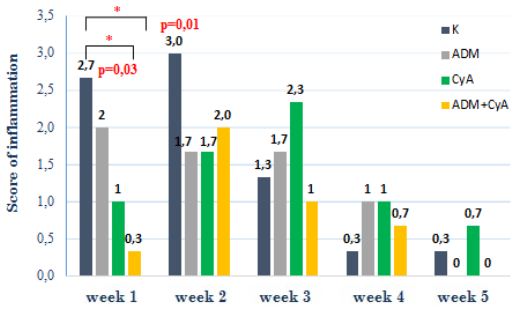

Figure 6: Inflammation scores of the groups

After the 4th week, the obliteration of the grafts showed significant difference between the control and the CyA treated groups. Only on the first week was the inflammation score significantly different between the control and CyA ($p<0.03$) and between the control and the ADM+CyA groups ($p<0.01$). Given, that the adrenomedullin did not show significant effect in all analyses, the experiment did not achieve the previously expected results.

II. 3D EXPERIMENTS

Because of the limitation of the BOS mouse model and because the first set of animal experiments did not achieve the previously expected results, we started another *in vitro* experiment with 3D lung tissue model in collaboration with the University of Pécs, Department of Pharmaceutical Biotechnology.

3D tissue models were set up. 6 different 3D cell cultures (Lonza, Basel, Switzerland) were analyzed at 24 and 48 hours after the set up (**D1**: donor 1; **D2**: donor 2):

1. **SN-D1:** epithel (SAEC) – **D1** + fibroblast (NHLF) – **D1**
2. **SH-D1:** epithel (SAEC) – **D1** + endothel (HMVEC-L) – **D1**
3. **SN D1/D2**: epithel (SAEC) – **D1** + fibroblast (NHLF) – **D1** + fibroblast (NHLF) – **D2**
4. **SH D1/D2:** epithel (SAEC) – **D1** + endothel (HMVEC-L) – **D1**+endothel (HMVEC-L) -**D2**
5. **SN-D2:** epithel (SAEC) – **D1** + fibroblast (NHLF) – **D2**
6. **SH-D2:** epithel (SAEC) – **D1** + endothel (HMVEC-L) – **D2**

The supernatant and lysated cells of the 3D tissues, and tissue specimens in a fixative solution were frozen at -80°C until further analysis. Taqman array were used for gene expression analysis of aquaporin (AQP1, AQP3, AQP5, AQP4), surfactant (SFTPA1, SFTPC), cadherin (CDH1, CDH2) and cytokines. The results of the gene expression are shown on **Figure 7**.

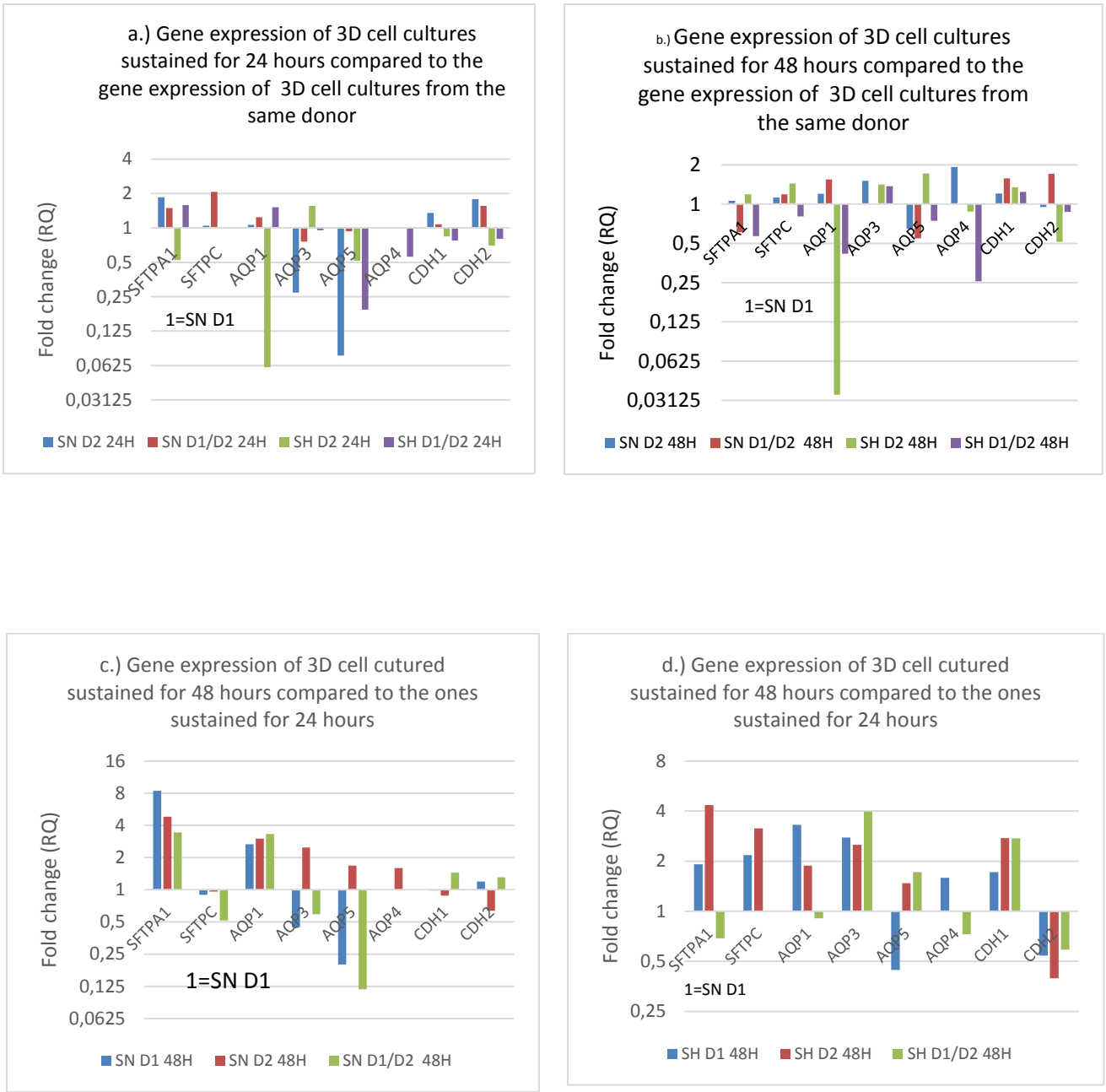

Figure 7: Taqman array gene expression analysis of aquaporin (AQP1, AQP3, AQP5, AQP4), surfactant (SFTPA1, SFTPC), cadherin (CDH1, CDH2) levels 24 and 48 hours after the set up.

Taqman array was used for gene expression analysis of proteins typically found in differentiated cells. The expression of cytokines, chemokines and growth factors was analyzed by Taqman immune array. According to the results, gene expression did not relate to the presence of the chimerism. Expression of some of the genes exceeded the non-chimera's gene expression, but some genes showed decrease. Similar results were obtained in case of expression of cytokines, chemokines and growth factors, the values did not correlate with the

presence of chimerism. Based on these results, no clear conclusion could be drawn with respect to chimerism. To establish a clear correlation, further examination is required, but this goes beyond the limits of this project. However, based on these results, we can state that in 3D cell models, which are being used by more and more researchers across the world, chimerism may lead to significant differences in results, thus causing incorrect conclusions.

III. CLINICAL PART OF THE STUDY

After receiving the ethical permission (04.05.2014. number of ethical permission: 1817/2014) our workgroup started to organize the human sample collecting at the National Institute of Oncology, Department of Thoracic Surgery. We have collected human blood and tissue samples from healthy individuals who underwent thoracic surgery because of recurrent spontaneous pneumothorax (PTX). These samples belonged to the control group. Bronchoalveolar lavage (BAL) and blood samples from Hungarian lung transplantation donors (after modifying 89/2015 ETT TUKEB ethical permission) were also a part of the control samples. As the number of the collected samples is insufficient for a comparison study, we are still collecting the samples from patient who underwent LTx and PTX. By 2020 we expect to have a reasonable number of sample of LTx patients which can serve as a basis for a more detailed analysis of the role of adrenomedullin in bronchiolitis obliterans syndrome. Hopefully, these results will be eligible for a peer-reviewed publication.