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Mesenchymal cells isolated from the airways of BOS patients as targets of innovative drug-loaded nanoparticles

Emanuela Cova[1], Miriam Colombo[2], Monica Morosini[1], Simona Miserere[1], Simona Inghilleri[1], Sara Magni[1], Davide Prosperi[2], Federica Meloni[1].
[1]Department Respiratory Diseases, IRCCS San Matteo, Pavia, Italy; [2]Department of Biotechnology and Bioscience, University of Milano Bicocca, Milan, Italy

Our group has recently shown that proliferating mesenchymal cells (MC), obtained from BAL of BOS patients, express CD44 and that this expression correlates with mTOR expression and with in vitro proliferative rate. By these results we have designed an innovative approach based on biocompatible nanoparticles loaded with the mTOR inhibitor everolimus and functionalized with anti CD44 MoAb, for the selective targeting to the specific cells (targNP). Fluorescent labeled targNP have been used to assess cell uptake by confocal microscopy. Cell apoptosis/death (annexin V/7AAD) and proliferation (CFSE) were evaluated by flow cytometry. We used primary MC isolated from 2 BOS patients (grade 1 and 2) with the following phenotype: BOS 1: 85% CD90+ of which 33% co-expressing CD9; BOS2: 93% CD90+ with 38% co-expressing CD146, 25% CD9, and 38% both CD146 and CD9. Both MC samples were negative for CD45RO and CD34 and positive for CD44 (98%). TargNP were shown to adhere to membrane within 15 min and completely enter into the cells after 45 min. Drug free nanoparticles, as control, were completely inert. TargNP treated cells showed a significantly higher mean rate of annexin V at 8 h (17,4 versus 4,5%) and 24 h (42,15 versus 5,3%) respect to control cells while mean 7AAD expression at 24 h was 4,1 versus 1.3%. Likewise, cell proliferation was significantly inhibited at 24 and 48 h (mean: 41 and 37.2%, respectively). This is, by far, the first proof of concept that an innovative approach based on drug coated NP can be used to selectively address MC which proliferate in the airways. Further in vitro and in vivo studies will investigate possible efficacy of this new treatment strategy.

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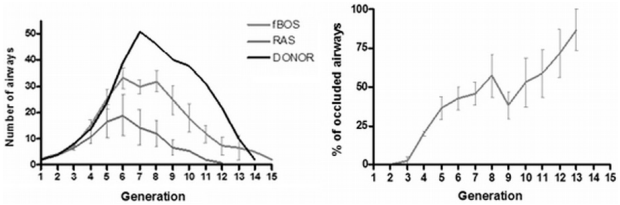
Structural differences in airways during chronic rejection after lung transplantation: A (micro)-CT analysis

Stijn Verleden[1], Stijn Willems[1], David Ruttens[1], Robin Vos[1], John McDonough[2], Annemie Vaneylen[1], Erik Verbeken[1], Johny Verschakelen[1], Dirk Van Raemdonck[1], Benoït Rondelet[3], Christiane Knoop[3], Marc Decramer[1], James Hogg[2], Geert Verleden[1], Bart Vanaudenaerde[1]. *[1]Pneumology, KU Leuven & UZ Gasthuisberg, Leuven, Belgium; [2]James Hogg Research Center, Sint-Paul's Hospital, Vancouver, Canada; [3]Pneumology, Erasme University Hospital, Brussels, Belgium*

Chronic rejection is a major problem after lung transplantation (Tx) and is accepted to be a small airway disease. Recently a distinction between an obstructive (fBOS) and restrictive phenotype (RAS) has been made. We aimed to investigate structural differences.

Human explant lungs from 5 fBOS and 3 RAS patients were collected during reTx, were air-inflated to TLC, frozen solid in liquid nitrogen vapor and frozen specimens were examined by HRCT. Unused donor lung (n=1) served as control. The lungs were kept frozen while cut in 2cm slices and cylinders of 1.4cm diameter were removed. These were fixed at -20°C in a solution of acetone/1% gluteraldehyde and warmed to 25°C, dried and scanned with microCT (skyscan1172, Belgium). HRCT images showed that RAS lungs displayed a lower number of airways starting at generation 5. In patients with fBOS >50% of the airways get obstructed at generation 6 (figure), mainly in airways <2mm (82%). MicroCT analysis shows a lower number of terminal bronchioles in RAS patients (2/ml of tissue vs 4.5/ml in fBOS and 5/ml in control, $p<0.0001$) and a lower average cross-sectional area of remaining terminal bronchioles (110mm^2 vs 178 in fBOS vs 175 in control).



In conclusion: in RAS lungs, both airways>1mm and terminal bronchioles shrink and/or disappear while in fBOS lungs, the airways become obstructed. There was no difference in terminal bronchioles between fBOS and normal lung.

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Th17 clones in BOS evaluated by enzyme linked immunospot assay

Simona Miserere, Rita Di Domenica, Monica Morosini, Anna Maria Grasso, Emanuela Cova, Federica Meloni. *Respiratory Diseases, Policlinico San Matteo and University of Pavia, Pavia, Italy*

IL-17- and Th17-associated cytokines have been linked to the development of acute and chronic rejection after lung transplantation in both animal models and humans. An increase in IL17 mRNA expression and of IL17 levels in BAL have been described in LTR with BOS and during AR episodes in comparison with Stables Recipients. On the other hand a decrease in IL10-producing clone number and in Treg cell frequency has been described in BOS patients.

Aim of the present study was to assess with a feasible method the balance between IFN-gamma/IL17- producing clones and IL10-producing cells/Treg cells in the peripheral blood of 26 LTR (13 stable recipients, 13 BOSpatients). IFNgamma IL17 and IL10 producing clones were assessed by ELISPOT. $CD4^+CD25^{high}CD127^{dim}$ were assessed by flow-citometry. A significant increase of IL17 and IFN-gamma producing cells in the peripheral blood was observed in patients who developed BOS (p= 0,03 and 0,04 respectively) while Treg cell count decreased significantly (p= 0,002) and IL10 showed a non significant trend toward a decrease. Moreover the ratio between IL 17 and IL 10 or Treg cell count was significantly increased in BOS (0,85 vs 0,21; 10,4 vs 1,9 respectively) while IFNgamma/IL10 ratio did not significantly change.

In conclusion detection of IL17/Treg ratio in the peripheral blood of LTR represents a feasible and useful tool in the identification of patients at higher risk of BOS development.By this way the role of Th17 axis in BOS pathogenesis is further confirmed.

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Mechanistical differences of chronic lung allograft dysfunction phenotypes in lung transplantation

Monika I. Suwara[1], Bart M. Vanaudenaerde[1,2], Stijn E. Verleden[2], Lee A. Borthwick[1], Robin Vos[2], Nicola J. Green[1], Chris Ward[1], Dirk E. Van Raemdonck[2], Paul A. Corris[1], Derek A. Mann[3], Geert M. Verleden[2], Andrew J. Fisher[1,3]. *[1]Lung Immunology Group, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom; [2]Leuven Lung Transplantation Unit, Katholieke Universiteit Leuven and University Hospital Gasthuisberg, Leuven, Belgium; [3]Fibrosis Research Group, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom*

Purpose: The neomacrolide azithromycin, is now widely used in the treatment of Bronchiolitis Oblierans Syndrome after lung transplantation. However, only a proportion of patients respond by improving their lung function. This study aimed to evaluate differences in airway microenvironment between azithromycin responsive (>10% improvement in FEV_1) and azithromycin resistant BOS patients.

Methods: Bronchoalveolar lavage (BAL) from recipients identified as stable n=10 (control), azithromycin responsive n=10 and azithromycin resistant n=10 were evaluated for cell differential, IL-1α, IL-1β, IL-6, IL-8, TNF-α proteins. BAL was then added to primary bronchial epithelial cells (PBEC) and tested for viability by XTT assay.

Results: BAL neutrophilia (%) was increased in responders (56% $p<0.0001$) and non-responders (52.9% $p<0.0001$) compared to the control (0.8%). IL-1α, IL-1β, IL-6, IL-8, TNF-α were increased in both groups (all proteins <0.05) compared to the control. The levels of IL-1α, IL-1b and TNFa showed increasing trend in responders compared to non-responders. PBEC viability in response to BAL was reduced in non-responders (p=0.012) but not in the responders group (p=0.64). Moreover, there was a negative correlation between PBEC viability and IL-1α (p=0.042), IL-8 (p=0.0017), TNFα (p=0.039), IL-1β (p=0.045) and IL-6 (p=0.0453) concentrations.

Conclusions: Unlike in responders, where azithromycin blocks IL-17 T cell mediated neutrophilia, azithromycin resistant phenotype is associated with epithelial damage, inducing IL-1α, TNFα and IL-1β release. This suggests anti-IL-1/TNFα therapies could be considered for BOS patients who develop an azithromycin resistant phenotype.

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Results of a phase 2b multi-center, randomized, double-blind, placebo-controlled study of an RNAi therapeutic, ALN-RSV01, in respiratory syncytial virus (RSV)-infected lung transplant patients

Amy Simon[1], Allan Glanville[2], Martin Zamora[3], Tony Hodges[4], Selim Arcasoy[5], MIchael Musk[6], Urte Sommerwerck[7], John DeVincenzo[8], Verena Karsten[1], Shaily Shah[1], Jeffrey Cehelsky[1], Sara Nochur[1], Jared Gollob[1], Akshay Vaishnaw[1], Jens Gottlieb[9]. *[1]Clinical Research, Alnylam Pharmaceuticals, Cambridge, MA, United States; [2]Lung Transplant Unit, St. Vincent's Hospital, Darlinghurst, NSW, Australia; [3]Department of Medicine, University of Colorado at Denver Health Sciences Center,Aurora, CO, United States; [4]Heart and Lung Institute, St. Joseph's Hospital and Medical Center, Phoenix, AZ, United States; [5]Department of Medicine, Columbia University, New York, NY, United States; [6]Advanced Disease Lung Clinic, Royal Perth Hospital, Perth, WA, Australia; [7]Ruhrlandklinik, Universitatsklinikum Essen, GmbH, Germany; [8]Department of Pediatrics and Molecular Sciences, University of Tennessee Center for Health Sciences, Memphis, TN, United States; [9]Innere Medizin, Medizinische Hochschule Hannover, Hannover, Germany*

RSV infection after lung transplantation is an independent risk factor for the development of bronchiolitis obliterans syndrome (BOS). ALN-RSV01 is a small interfering RNA targeting the RSV nucleocapsid gene that is critical for viral replication. Previously, we performed a Phase 2 randomized, double-blind, placebo (PBO)-controlled trial in 24 RSV-infected lung transplant patients administering

aerosolized ALN-RSV01 or PBO for 3 days. The primary endpoint of safety and tolerability was attained. In addition, there was a significant reduction in the secondary endpoints of incidence of new or progressive BOS at day 90 (p=0.027) and patient's symptom scores in the ALN-RSV01 group compared to PBO. To extend these results, we performed a Phase 2b multi-center, multinational, randomized, double-blind, PBO-controlled trial in RSV-infected lung transplant patients in which the primary endpoint was the effect of ALN-RSV01 on the incidence of new or progressive BOS at Day 180. Secondary endpoints included the impact of ALN-RSV01 on symptom scores, antiviral activity and safety. RSV positive subjects were randomized (1:1) to receive either aerosolized ALN-RSV01 or PBO for 5 days, alongside the hospital's standard-of-care. Subject stratification to treatment arms was based on two binary factors: 1) time from symptom onset to treatment start and; 2) pre-infection BOS grade. Of the 3,985 subjects prescreened at 33 centers, 218 were RSV positive, of which 87 were randomized. Enrollment is completed and subjects are now in the follow-up phase. Final study results will be presented at this meeting.

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BAL neutrophil levels in a randomised controlled trial of azithromycin therapy in bronchiolitis obliterans syndrome post lung transplantation

Chris Ward[1], Gail Johnson[2], Victoria Ryan[3], Therese Small[2], James Lordan[2], Andrew Fisher[1], Gerard Meachery[2], Paul Corris[1]. *[1]Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom; [2]Institute of Transplantation, Freeman Hospital, Newcastle upon Tyne, United Kingdom; [3]Institute of Health and Society, Newcastle University, Newcastle upon Tyne, United Kingdom*

Open studies have reported an improvement in FEV1 in some BOS patients treated with Azithromycin (Azith). We recently demonstrated that Azith is superior to placebo treatment, improving FEV1 in patients with BOS. It is suggested that a BAL neutrophil reduction is associated with treatment benefit. We have investigated this in our trial.

Methods: A prospective, randomised double blind placebo controlled study of Azith 250mg o.d. or placebo on alternate days, in BOS patients. The primary outcome was change in FEV1 at 12 weeks and a secondary outcome BAL neutrophil %.

Results: 46 patients were randomised (23 Azith, 23 placebo) stratified for pre op diagnosis of suppurative disease and operation (single or bilateral lungs). Of the 46 ITT patients, baseline and final visit BAL were available for 28 (13A, 15P). In these patients the baseline % neutrophils, median (IQR), in the Azith group was 17 (4 to 69%) and in the placebo group 15 (2 to 56%). The baseline to final visit change in % neutrophils varied, with around half the patients in both treatment arms showing an increase from baseline and half a decrease. There was a median increase from baseline in the Azith group of 10%, IQR (–10% to 18%) and in the placebo group a median decrease from baseline of –1% (–8% to 5%). These changes were statistically non significant (P=0.4 and 0.7 respectively, 1-sample Wilcoxon).

Conclusion: Azith is superior to placebo improving lung function in patients with BOS. In the same patients, whilst BAL neutrophil % was high, we observed no consistent fall with Azith. This may suggest the mechanism of benefit does not rely on a quantitative reduction in neutrophils.

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ENDOXY – Endothelialization of a gas permeable membrane for the development of a biohybrid lung assist device

Christian G. Cornelissen[1], Maren Dietrich[2], Dirk A. Frechen[1], Jörg Sachweh[2], Stefan Krüger[1], Stefan Jockenhoevel[2]. *[1]Dept. for Internal Medicine – Section for Pneumology, RWTH Aachen University Hospital, Aachen, Germany; [2]Dept. for Tissue Engineering & Textile Implants, Institute for Applied Medical Engineering, Helmholtz Institute of the RWTH University Hospital, Aachen, Germany*

Introduction: Extracorporeal membrane oxygenation's (ECMO) limited hemocompatibility, the activation of the coagulation system and the complement system, plasma leakage and protein deposition hamper mid- to long-term use and have constrained the development of an implantable lung assist device. In a Tissue Engineering approach, lining the blood contact surfaces of an ECMO device with endothelial cells might overcome these limitations.

Methods: ENDOXY - a multifunctional oxygenator test device was developed to endothelialize gas permeable membranes and to test the gas transfer through it. Endothelial cells were preconditioned by applying shear stress in the test-system and monitored via a life cell imaging device. Cell coated membranes were incubated with blood and characterized by immunohistological staining.

Results: Cells seeded on gas permeable membranes grew to confluence and showed characteristic endothelial morphology and aligned with shear stress without observable cell detachment. Fixed samples stained positive for endothelial markers. No corpuscular blood constituents resided on the endothelialized surfaces after static or dynamic incubation with blood, suggesting promising hemocompatibility.

Conclusion: The development and initial application of the ENDOXY system is a first step toward the development of a biohybrid lung assist device.

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LSC 2012 Abstract – Lung tissue engineering: generation and characterization of decellularized lung scaffolds for stem cell differentiation

Sharareh Shojaie[1,2], Martin Post[1,2]. *[1]Department of Physiology, University of Toronto, Canada; [2]Physiology and Experimental Medicine, Hospital for Sick Children, Toronto, Canada*

The interaction of stem cells with the surrounding matrix environment is crucial for cell fate. The development of biomatrices that recapitulate the in vivo environment is a key component to driving differentiation of pluripotent cells into lung endoderm precursors. We investigate whether decellularized lungs with intact matrix composition can promote the differentiation of embryonic stem cells (ESC) into distal lung epithelial cells. Rat cadaveric lungs were decellularized by sequential tracheal lavages and retrograde pulmonary arterial perfusion using a range of physical, chemical, and enzymatic treatments. Histological staining, immunofluorescence, electron microscopy, and tensile testing have confirmed decellularization and preservation of matrix proteins. Murine ESC (Foxa2/CD4; Bry/GFP cells) were seeded onto scaffolds following endoderm induction using activin, and analysed for lung lineage marker expression. Seeded ES cells maintained FoxA2 expression and adopted an epithelial-like tubular organization. This demonstrates the ability of acellular lung scaffolds to support the adherence, proliferation, and potential differentiation of murine embryonic stem cells. Current studies are analysing their potential as viable scaffolds for the unidirectional differentiation of human endoderm-induced ESC (Hes2 cell line) into distal lung epithelial cells.