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STUDY OF ACCEPTANCE/REJECTION MECHANISMS IN LUNG ALLOGRAFT RECIPIENTS AND DEVELOPMENT OF A NEW THERAPEUTIC APPROACH BASED ON NANOTECHNOLOGY

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INDEX

BACKGROUND

Lung transplantation is the only therapy that can prolong the survival of people with end-stage chronic lung disease no longer responsive to medical or surgical treatment. Pathologies eligible to lung transplant are: pulmonary emphysema associated or not with deficit1-antitrypsin deficiency, idiopathic pulmonary fibrosis o other progressive interstitial lung diseases, cystic fibrosis and bronchiectasis, primary pulmonary hypertension and pulmonary hypertension secondary to congenital heart disease, such as Eisenmenger's syndrome, langerhans cell lung histiocytosis , lymphangioleiomyomatosis (LAM), IV stage sarcoidosis or connective tissue diseases (Judson, 1998; O'Brien et al., 1995; Frost et al., 1993).

The evaluation criteria for the inclusion in the waiting list of patients with advanced pneumopathies were identified by Orens and coll. in 2006 and subsequently reviewed (Orens et al., 2006; Shah et Orens, 2012). The International Society for Heart and Lung Transplantation has recently modified general indications and specific indications for the timing of referral at the transplant center and for listing (Weill et al., 2014).

The greatest obstacle to the success of transplants has always been represented by acute or chronic rejection. The recipient's immune system reacts to the presence of non-self molecules, activating an immune response that leads to organ rejection. The non-self molecules (either HLA class I or II or non-HLA antigens) are presented to the recipient alloreactive T lymphocytes in different ways: direct, indirect or semi-direct (Caballero et al., 2006). The allospecific reaction is the best known in the context of lung transplant and can be either cellular or humoral.

The lung rejection is thus based on histo-pathological characteristics, the main involved pathway, its reversibility and on the basis of the anatomical district involved (vascular, interstitial, alveolar or bronchiolar :

1. Hyperacute rejection
2. Acute cellular or humoral rejection
3. Chronic Rejection in the context of a Chronic Dysfunction of the Pulmonary Allograft (CLAD): which is further sub-phenotyped as obstructive (Obliterative Bronchiolitis Syndrom-BOS) or restrictive (Allograft Restrictive Syndrome-RAS).

While acute cellular rejection either vascular or bronchiolar, frequent in the first year post transplant, are currently controlled by immunosuppressive treatment (high dose of steroid course, anti-lymphocyte Ab), chronic rejection remains the main limitation to long-term survival of lung transplant recipients. Based on current revision of CLAD definition (under publication at the moment), the chronic (sustained) decrease of lung function unresponsive to immunosuppression or azithromycin, not due to acute infection, acute cellular or Ab mediated rejection can be defined as

CLAD. CLAD is an umbrella term that includes several graft and extra-graft disorders. Once all extra-graft disorders as well as other possible graft causes (such as chronic infections, pleural problems, etc) are excluded, chronic rejection may be diagnosed and sub-phenotyped according to pulmonary function tests in obstructive or restrictive. Bronchiolitis Obliterans Syndrome (BOS) is the major phenotype of chronic rejection whose histological counterpart is the obliterative bronchiolitis (OB, a first inflammatory obliterative lesion becomes fibroproliferative and affects the small airways).

The main characteristics of obstructive and restrictive phenotype are shown in Table 1.

	BOS	**RAS**
Time	Early (within the first year from Tx) or late (2-3 years from Tx). Prevalence almost 70% at 10 years from Tx	Early or Late. Represents almost 20% of CLAD.
Physiopathology	Obstructive (persistent, mostly irreversible, progressive decline in FEV1 compared to the best value post-Tx)	Restrictive (persistent decline in FEV 1 of >20% compared to the best post Tx value and a decline in total lung capacity of >10% compared to baseline)
HRCT Imaging	Bronchiectasis and air trapping signs	Pattern of persistent interstitial/upper lobe fibrosis
Hystopathology	Obliterans Bronchiolitis (OB): scarring process affecting the small airway; it may originate either from a chronic inflammation over several years or from a direct and severe insult of the respiratory epithelium over a period of several months	Fibrosis, thickening of the intralobar septa and/or of the pleura Acute alveolar damage may be present.
Clinical hystory	Usually progressive; possible stabilization in few cases	Always progressive. Prognosis much worst than BOS

Table 1.

In this thesis, I have mainly addressed several aspects of BOS: risk factors, role of the regulatory immune system and therapy.

Risk factors for the development of BOS

A recent review of the literature data by a working group of the International Society for Heart and Lung Transplantation (ISHLT) (Meyer et al. 2014), highlighted several risk factors involved in the development of BOS: recurrent episodes of acute rejection and presence of high allospecific cellular and humoral reactivity (Palmer et al. 2002) seem to be the major risk factor for BOS development (Bando et al. 1995, Burton et al. 2008). Other known risks factors for the development of BOS include the severity of ischemia-reperfusion injury, the presence of significant (abnormal in degree) gastro-esophageal reflux (Goettlieb et al, 2009 and Khalid et al., 2013), BAL neutrophilia (Zheng et

al, 2006) and viral, bacterial or fungal infection (in particular CMV graft infections/diseases and chronic graft colonization by Ps aeruginosa or fungi or specific respiratory viruses). (Chaparro et al., 1994; Magnusson et al., 2013; Vu et al., 2011; Gottlieb et al., 2009; Billings et al., 2001; Vilchez et al., 2001; Neofytos et al, 2010). From different centers have been reported positive results for the reduction of the incidence of BOS in patients undergoing long-term prophylaxis of CMV infection (Patel et al., 2012; Paraskeva et al., 2011; Kotton et al. , 2010; Valentine et al., 2008; Hodson et al., 2008). It has also been shown that BOS is associated with the alteration of the post-transplant lung microbiome, with an increase in bacterial load (Willner et al., 2013) and a shift towards more proinflammatory and profibrotic species. Distal airway microbiome has been evaluated in lung transplant in the view that low airwaysrepresent an echosystem where host inflammatory cells modulates their activity in coordination with microbial communities. In BOS an increase in relative abundance in of Protobacteria, Firmicules and Bacteroides in distal airways with respect to NON BOS patients has been described (Bernasconi et al 2016). More recently, Mouraux et al demonstrated that catabolic-remodeling associated species (Staphylococcus, Corinebacterium, Stenotrophomonas and Haemofilus) were associated to a higher degree of inflammation and to a matrix remodeling gene expression profile in macrophage /fibroblasts co-colture. (Mouraux et al 2018)

Physiopatology of BOS

The exact pathogenesis of BOS is not well understood, but is thought to be the result of a chronic immune and inflammatory reaction that involves an exaggerated reparative fibrotic response. Chronologically the pathogenetic process can be divided into three phases:

- First Phase: there are lesions of the bronchial or bronchiolar epithelial lining tissue, caused by a primary insult that can be isolated and severe or repetitive and milder. This damage could be due to an all-specific reaction (cellular or humoral against graft antigens) or to an inflammatory or cytopathic injury caused by infectious agents, gastroesophageal reflux or other insults.
- Second phase: it is the so-called "cellular bronchiolitis" or "active": inflammatory cells (lymphocytes, macrophages, neutrophils) infiltrate bronchial wall and produce cytokines, chemokines, prostaglandins, leukotrienes that establish a chronic inflammatory response. Neutrophils are activated by Th17 and arrive in the bronchiolar lumen. The role of lL-17 in the pathogenesis of BOS is well codified and demonstrated by numerous studies (Nzujaht et al., 2012). One of the main mediators of airway inflammation in patients with BOS is IL-8, a

neutrophil specific cytokine, belonging to the CXC chemokine family and produced by macrophages and smooth muscle cells. In addition to neutrophils, the T and B cells, monocytes and macrophages also come into play at this stage. Resident alveolar macrophages are an important source of profibrotic factors (TGF-β) and induce EMT, differentiation of resident and circulating stem cells myofibroblasts (Sato et al., 2008 Sivakumar et al., 2008).

- Third phase: this phase is represented by an exaggerated reparative reaction. Following the inflammation phase, a fibroproliferative phase occurs, driven by growth factors such as the Platelet Derived Growth Factor (PDGF), the Insulins-Like Growth Factor (IGF), the Fibroblast Growth Factor (FGF), the TGF-β and the TNF-α. All these factors lead to a marked proliferation of myofibroblasts and with consequent deposition of matrix proteins such as collagen I, II and IV thus leading to a fibrotic occlusive lesion into the small airways lumen (Belperio et al., 2009; Boehler et al., 2003).

<u>BOS Diagnosis</u>

The algorithm for clinical evaluation of suspected bronchiolitis obliterans syndrome (BOS) has been published in a ISHLT/ATS/ERS consensus document from 2014 (Meyer et al, 2014) (figure 1) and is currently under re-definition by a panel of expert belonging to ISHLT, but no-significant changes in BOS can have a very variable development and progression:

• sudden onset with rapid decline in lung function;
• insidious onset with a slow and progressive decline in lung function over time;
• rapid initial decline of FEV1 followed by a prolonged period of stability (Nathan et al., 1995).

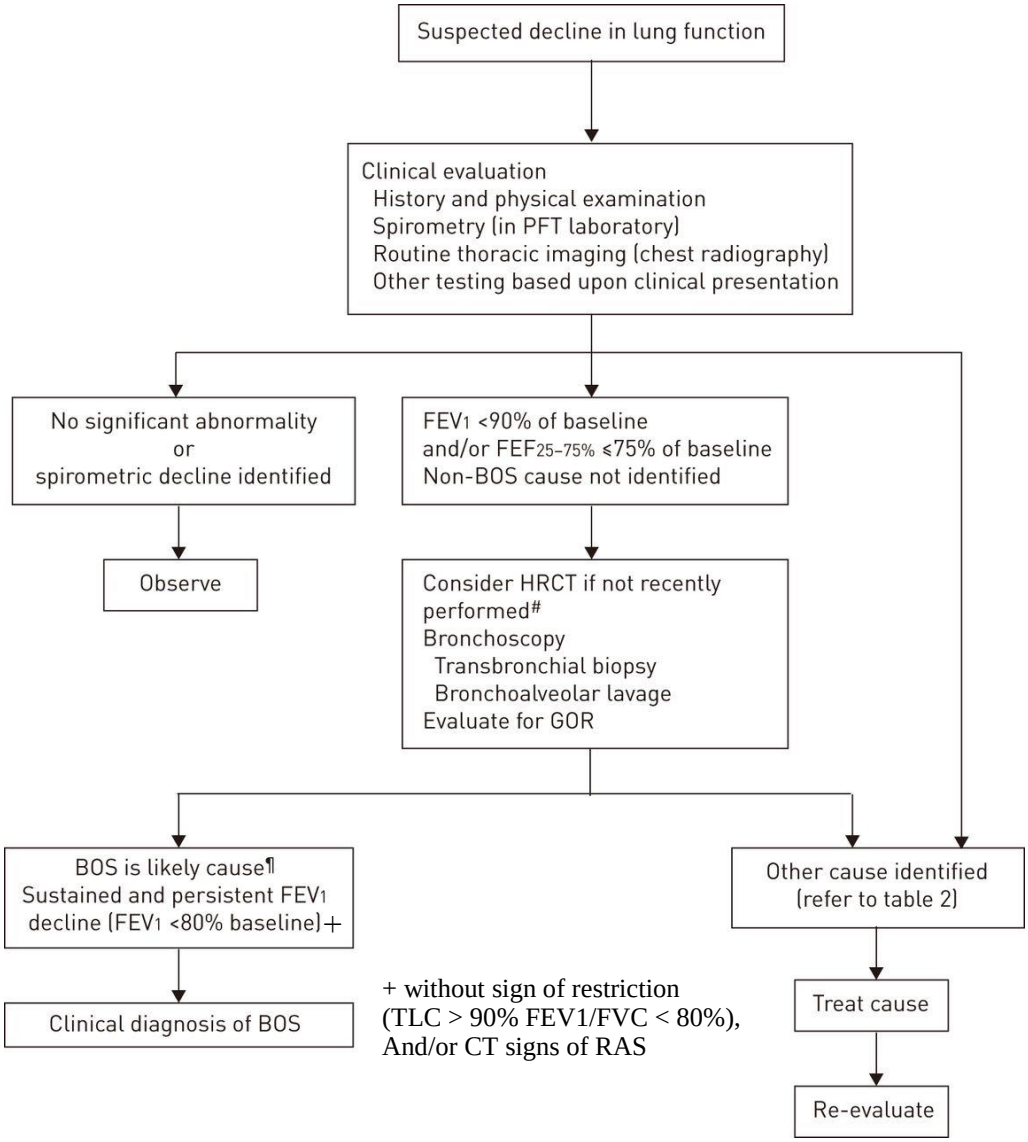

Figure 1. Diagnostic algorithm for BOS adapted from Meyer et al Eur Respir J 2014;44:1479-1503.

The functional staging of the BOS allowed to divide the patients into different categories according to the different degree of graft dysfunction and to predict prognosis (Figure 2). However, this classification does not provide precise therapeutic indications since the evolution of the pathology is highly unpredictable (Mc Giffin, 2002); therapeutic choices are not only based on the functional class but also take into account the many risk factors.

In 2002 the classification proposed by ISHLT was revised with the insertion of a 5th stage defined pre-BOS or BOS potential stage (BOS 0p) (Estenne et al., 2002). The 0p stage has been regarded as relevant as it acts as an alarm signal for the development of BOS. The stages of BOS are summarized in Table 2

BOS stage	FEV1 post LTx (compared to Best FEV1 post Tx)
0	> 90%
0p	90-81%
1	80-66%
2	65-50%
3	< 50%

Table 2 stages of BOS according to FEV1 decline

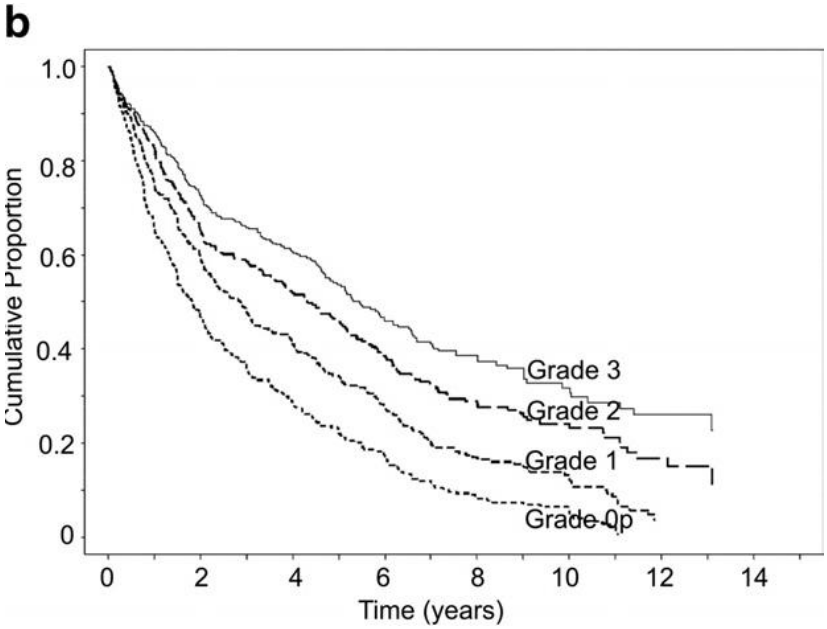

Figure 2 f-BOS determines prognosis according to BOS grade. Burton et al. JHLT 2007;26:681-686

A key aspect in the management of patients who have undergone organ transplantation is the modulation of immunosuppressive treatment both to prevent rejection and to minimize infectious risk or systemic toxicity. Although much progress has been made in the development of therapeutic strategies to improve patient and transplant survival, to date there are still adverse events associated with many immunosuppressive drugs and their long-term use. It is still difficult to adapt the care; tailored to each individual reactivity/infection risks in order to balance the risks and benefits of drug treatment. The toxicity associated with the use of calcineurin inhibitors and steroids has led to the drafting of numerous protocols aimed at reducing immunosuppressive therapy. One of the most important challenges in reducing immunosuppressive treatment is how quickly the effect on the patient's immune system can be assessed. The immune system, in fact, is determined

by the dynamic interaction of positive and negative regulatory responses that are dependent on the individual response to drugs, general clinical conditions, diet, genetic context, gender, age. In fact, a reduction in immunosuppressive therapy is only possible if there is a way to monitor and reliably identify patients at immunological risk. Since 2002, the Food and Drug Administration (FDA) has approved an immune function assay that measures the concentration of ATP in CD4 + T cells stimulated with phytohamagglutinin (PHA), named Immuknow assay. This assay, which monitors the immunosuppressive degree of kidney, liver, lung, intestine and pancreas transplant patients has proven to be very useful above all for the prevention of infections (ref), which are an important cause of mortality and morbidity.

Therapy of BOS

Unfortunately, in the case of lung transplant no newly immunosuppressive strategies have resulted in an improvement in survival, bringing to BOS prevention.

However, it is important to halt or slow down the decline of FEV1: therefore the therapy will have to use combinations of drugs and treatments that prevent the onset of the disease or stabilize it once it has established itself.

Intensified pharmacological immunosuppression has little effect on established BOS in the absence of confounders such as acute cellular rejection, antibody-mediated rejection or lack of BAL neutrophilia. On the other hand, these strategies tend to increase infection risk and cause a more rapid progression of lung function decline

Thus in recent years, few pharmacological strategies has been tested in order to immunomodulate these lung recipients and induce a higher acceptance of the graft, decreasing the progression of BOS. Azithromycin, generally administered orally at 250 mg per day for 5 days and then 250 mg three times per week for at least 3 months has proven to be effective in prevention of BOS onset (Verleden et al 2004, Corris et al 2015); or using extracorporeal photopheresis (FEC), an immunomodulatory treatment based on leukapheresis, has been reported by retrospective study to slow down the graft function decline in nearly 60% of BOS treated recipients.

In addition, conventional treatments for BOS are poorly effective mainly due to their systemic administration combined with their non-targeted nature, resulting in unpredictable biodistribution, insufficient drug accumulation in the thorax, limited efficacy, and systemic toxicity.

On the basis of this therapeutic scenario, our group has previously explored the efficacy of azithromycin and ECP in BOS outcomes and the designed a new therapeutic approach based on nanotechnology. In a previous study by our group (Cova et al, 2015) targeted gold nanoparticles

(GNPs) loaded with the mammalian target of rapamycin inhibitor everolimus, specifically decorated with an antibody against CD44 were engineered. CD44 was chosen as ideal receptor for NP targeting since it is a surface receptor expressed at high rate (>85%) by primary Mesenchimal Cells (MCs) isolated from bronchoalveolar lavage of BOS patients and by activated T-lymphocyte.

NPs were loaded with everolimus, a synthetic drug which inhibits the mammalian target of rapamycin (mTOR), a kinase that regulates cell growth and metabolism in MCs. (Figure)3

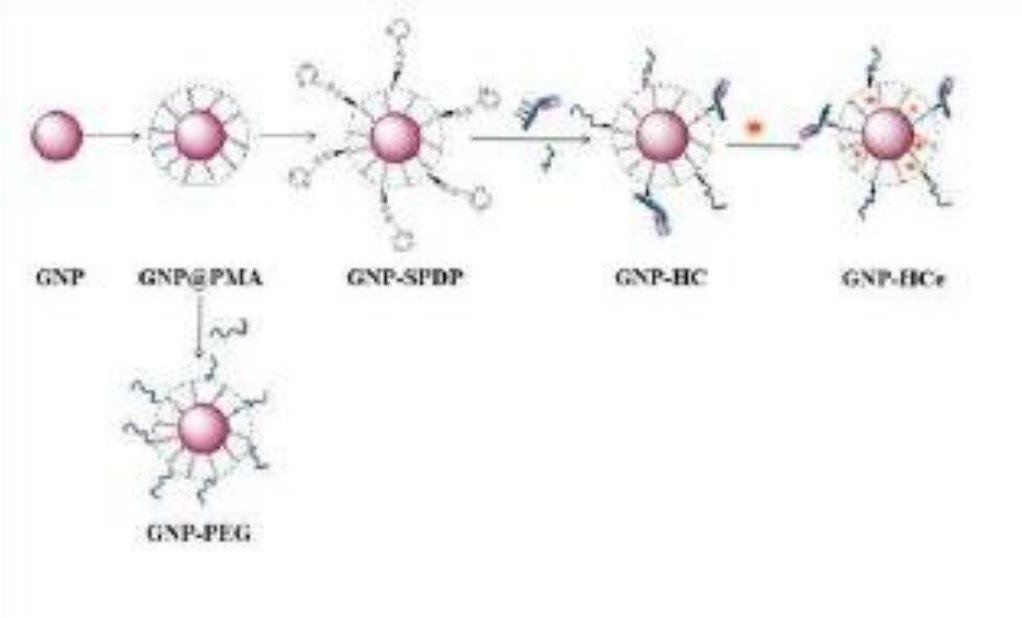

Figure 3. Step sequence of GNPs preparation.

In a previous work, GNPs showed to be internalized by MC only if CD44 loaded, within 1 hour of incubation (figure 4). In addition, GNP-HCe (decorated with antiCd44 and functionalized with everolimus) significantly inhibited MCs proliferation and induced higher apoptosis rate in vitro, but also the effect was higher and longer lasting compared to treatment with the drug alone. (Cova et al 2015)

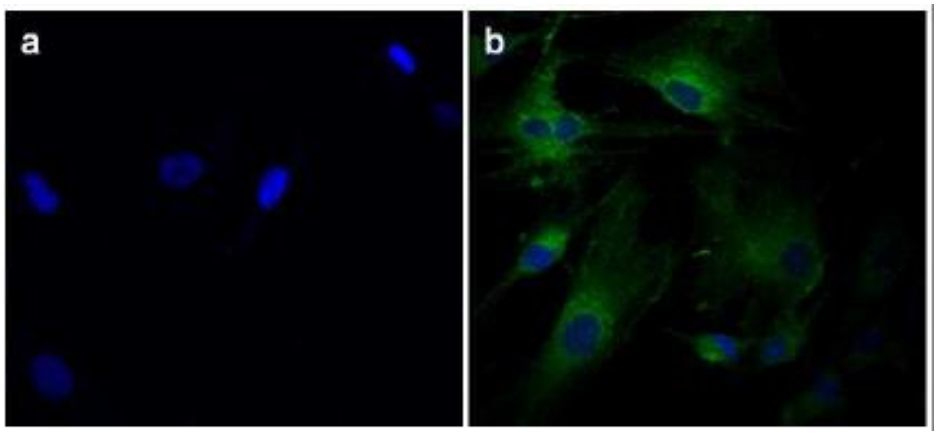

Figure 4. Confocal images showed no cellular signal in presence of non functionalized GNP (GNP-PEG) (A), while fluorescence signal is detected inside the cells after 1h incubation with functionalized GNP (GNP-HCe). (Cova et al 2015)

AIMS

This PhD project focuses on BOS , analyzing new risk factors, pathogenetic mechanism and identifying new innovative therapeutic approaches.

Aim 1: to find and study unknown risk factor which may have an impact on chronic rejection development and survival after lung transplantation. Many risk factor are already known to be relevant in BOS pathogenesis, such as recurrent episodes of acute rejection, CMV infection, gastro-esophageal reflux, time of ischemia of the graft and HLA mismatch. In the first year of this PhD, I have participated in an European multicenter study (enrolling all lung transplant recipients from our center in Pavia), where the role of another relevant risk factor was identified. The exposure of lung recipients to long-term residential and traffic air pollution, which were found significantly associated with CLAD occurrence and survival after lung transplantation (Ruttens, Piloni at al., 2017). During the PhD the 4-months research fellowship at Lung transplant Unit at Columbia University in New York a clinical research on the role donor lung pathological findings in conditioning clinical outcomes of lung transplant recipients were also assessed ("Donor lung pathological abnormalities and recipient outcomes", abstract ERS 2018).

Aim 2: to explore the immunological risk factors of BOS apart from the occurrence of Acute rejection. The exact pathogenesis of BOS is not well understood, but is thought to be the result of a chronic immune and inflammatory reaction that involves an exaggerated reparative fibrotic response. The role of the balance between allospecific immunity/tolerance towards graft antigens is still highly debated. Thus, we have decided to analyze the role of Regulatory T and B cell in the pathogenesis of BOS with two papers that confirmed the possible role of T-regulatory cells in the onset and evolution of BOS (Piloni et al., 2017), and highlighted that regulatory cells (B-regulatory cells) might as well participate in long-term lung graft acceptance mechanisms (Piloni et al, submitted). In addition, we have analyzed the effect of different immunosuppressive strategies, either as induction

or as maintenance regimen, on T cell reactivity and transplant outcome. In a paper, we compared patients from 3 different lung transplant centers in Europe who encountered different induction strategies (no induction, anti-thymocyte globulins and basiliximab) and we found that the blood lymphocyte count after LTx predicts one year mortality after lung transplant (Coiffard, Piloni et al, 2018). In another paper we have quantified the burden of chronic immunosuppressive treatment by means of the Immuknow assay, measuring the ATP production from peripheral blood CD4+T-cells in order to analyze T cell activation and correlate it with risk of infections and CLAD onset, either RAS or BOS (Piloni et al., 2016).

Aim 3: to explore the effectiveness of a newly designed a new therapeutic approach for BOS. Thus, our research group has recently designed and developed an innovative therapeutic approach of BOS, consisting in the use by local route of nanocarriers (Gold NanoParticles), loaded with specific anti-proliferative/immunosuppressive drugs and specifically targeted to disease cells (Fibroblasts/lymphocytes/macrophages) responsible of the pathological processes. During these years, we were able to prove that our bioengineered nanotools do not rise an inflammatory response and, under specific inhalator conditions, are non-toxic (Cova et al, 2017).

Finally, in collaboration with Columbia University we assessed lung distribution of aerosolized Gold nanoparticles in lungs of rats during mechanical ventilation ("Aerosolized gold nanoparticles as a vehicle for prolonged donor lung treatment", Abstract ERS 2018), a study which assess feasibility of this therapeutic strategy during ex vivo lung riperfusion.

MATERIALS AND METHODS

Patients

All patients included in the studies presented in this thesis have been submitted to lung transplant and followed up in the Respiratory Diseases department at IRCCS Policlinico San Matteo Foundation, a 1,200-bed teaching hospital where lung transplant program initiated in 1991. All patients has signed the informed consent form for the participation in these studies. All our patients undergo a similar clinical, functional, radiological, endoscopic and immunological follow-up, standardized according to international guidelines.

Our immune suppression protocol has undergone some changes over time: all patients transplanted between 2001 and 2007 were treated with a triple immunosuppressive regimen (cyclosporine, azathioprine, and prednisone), whereas patients transplanted since January 2008 received a modified standard triple regimen (tacrolimus, mycophenolate mofetil, and prednisone). In case of refractory acute rejection (AR), patients were switched from cyclosporine to tacrolimus and from azathioprine to mycophenolate mofetil. In the presence of documented renal dysfunction, patients were treated with low- dose tacrolimus plus everolimus. All patients underwent surveillance and on-need bronchoscopies; biopsy-proven episodes of AR (Stewart et al 2007) were treated with steroid boluses and, in case of AR recurrence or persistence, with a standard anti-thymoglobulin course and a modulation of the immunosuppressive regimen. BOS diagnosis and severity grades has been assessed according to published guidelines: briefly is a persistent, mostly irreversible, progressive decline in FEV1 (compared to the best value post-Tx) + /- typical CT findings (bronchiectasis and air trapping signs) (Estenne et al 2002, Verleden et al 2015 and 2015). The CLAD subtype RAS was retrospectively re-classified for patients diagnosed before 2013, according to functional (persistent decline in forced expiratory volume in 1 s (FEV 1) of >20% compared to the best post Tx value and a decline in total lung capacity of >10% compared to baseline) and radiological criteria (CT scan showing a pattern of persistent interstitial/upper lobe fibrosis) (Estenne et al 2002, Sato et al 2011, Verleden et al 2015). In case of a BOS 0p (FEV1 between 90 and 80% of best value post-Tx) or early RAS diagnosis, patients were prescribed a 3-month course of chronic low-dose azithromycin and were screened for gastro-esophageal reflux and anti-reflux medical treatment was maximized. In case of a further decline consistent with a CLAD diagnosis, since 2003, patients are referred to the Apheresis Unit for compassionate Extracorporeal Photopheresis treatment (Del Fante et al 2015). Our cytomegalovirus surveillance protocol is described in previous paper from our group (Lilleri et al 2013). All patients were submitted to the assessment of a complete peripheral immune phenotype at least twice a year.

Assessment of air pollution exposure

The residential addresses of all lung transplant patients were geocoded (ArcGIS10; www.esri.com). Subsequently they were linked with average levels of PM10 (µg·m–3), using an EU map of PM10 for 2007 based on land use regression, using predictor variables from EU-wide databases with satellite-derived particulate matter estimates, north–south trend, land use, roads and altitude (Beelens et al 2009). Particulate matter data for Switzerland were not available within this study, although we could quantify all other variables. Residential proximity to major roads or freeways and total length of roads within different buffers (50, 100, 200, 500 and 1000 m) around home addresses of patients were also estimated, as in Figure 5.

Figure 5. The visual presentation of the buffer zones (50m, 100m, 200m, 500m and 1000m) and their road length around a home address together with the distance to a major road and freeway.

Flow cytometric determination of peripheral Lymphocyte subsets

Flow cytometry was performed on a Beckman Coulter Navios using Kaluza software (Beckman Coulter). Briefly, 50 µl of fresh whole blood was incubated with the appropriate amounts of fluorochrome-labeled monoclonal antibodies CD45 APC Alexa Fluor 750, CD4 APC, CD69ECD, CD25 PE, CD127 PC5 for the T-reg and CD45 APC Alexa Fluor 750, CD4 APC, CD3 FITC, CD8 PE, CD56/16 PC5, CD19 PC7 for the lymphocytic population (Beckman Coulter) at room temperature in the dark for 15 min using appropriate mouse immunoglobulin isotypes as a control. Following incubation, 1 ml erythrocyte lysing solution was added to the samples and incubated under the same conditions for 20 min. In some samples, peripheral blood mononuclear cells (PBMC) were stained with CD4 APC, CD25 FITC, and CD127 Alexa Fluor 647 (Beckman Coulter), fixed and permeabilized, followed by intracellular staining with Foxp3 PE or control IgG1 (Human Regulatory T cell Staining kit, eBioscience) for 30 min. Finally, the cells were characterized by flow cytometry analysis.

Phenotype analysis of T-regulatory by flow cytometry

Peripheral blood assessment of regulatory T cell subsets has significantly evolved in the last years, even more so during the realization of this study. For this reason, we started the protocol performing the analysis of peripheral $CD4^{+}CD25^{high}CD127^{-}$ cell subset, but once commercially available, we also quantified FOXP3+ cells. Therefore, we could determine that peripheral $CD4^{+}CD25^{high}CD127^{-}$ T-reg cell subset included a mean of 93.15% (±4.34) FOXP3+ cells (Figure 1, illustrative case). For this reason, and in accordance with published evidence (Zhang et al 2015) we included $CD4^{+}CD25^{high}CD127^{-}$ cell determinations in the final statistical analysis, expressed this subset as absolute number (n°/µl peripheral blood) and named these cells $CD4^{+}CD25^{high}CD127^{-}$ T-reg cells.

Phenotype analysis of B-regulatory by flow cytometry

To characterize the phenotype of B lymphocyte subsets, 50 µl of heparinized venous blood was used for surface staining using antibodies against CD19 APC (Allophycocyanin; clone SJ25C1; BD Biosciences), CD24 PE-Cy7 (Phycoerythrin Cyanin 7; clone ML5; BD Biosciences)and CD38 PE (R Phycoerythrin; clone HIT2; BD Biosciences). After 15 minutes of incubation at room temperature in the dark, red blood cells were lysed with FACs Lysing Solution following the manufacture's protocol

(BD Biosciences). FACS analysis was performed by gating first on the lymphocyte and after on the CD19 B cell population. Acquisition and analysis were performed on a BD FACSCanto II (BD Biosciences).

Intracellular stain for IL-10 detection

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient-separation (Lymphoprep). For analysis of cytokine production, 1 x 10^6 cell/ml were stimulated with ODN 2006 Type B CPG (1 µg/ml; InvivoGen) and Recombinant Human CD40 Ligand/TNFSF5 (1 µg/ml; R&D Systems) for 72 h at 37°C and 5% CO_2. For the last 5 h were added Brefeldin A (5 µg/ml), PMA (50 ng/ml) and ionomycin (1 µg/ml) (Sigma Aldrich). Cells were washed and stained for CD19 APC, CD24 PE-Cy7 and CD38 PE and incubated for 20 minutes at room temperature in the dark. After membrane staining, the cells were fixed and permeabilized using the Lyse/Fix (BD Biosciences) and stained with anti-human IL-10 FITC (Fluorescein isothiocyanate; clone B-N10; IQ Products) for 30 minutes at room temperature in the dark. Acquisition and analysis were performed on a BD FACSCanto II (BD Biosciences).

ViraCor-IBT Immunoassay

Immune cell function was measured by the IMK assay (ViraCor-IBT, Columbia, MD), following the manufacturer's instructions. Briefly, sodium heparin anticoagulated blood was incubated for 15–18 hours with phytohemagglutin at 37°C in an incubator with 5% CO_2. CD4+ cells selected with the use of antibody-coated magnetic beads were washed and lysed to release intracellular adenosine triphosphate (iATP). The iATP release was measured by luminescence using a Luminescence reagent detection system. (Figure 6) The increased iATP from CD4 cells after *in vitro* stimulation defined 3 immunologic response zones: strong (>525 ng/mL), moderate (525-225 ng/mL), and weak (<225 ng/mL) (Kowalski et al 2006).

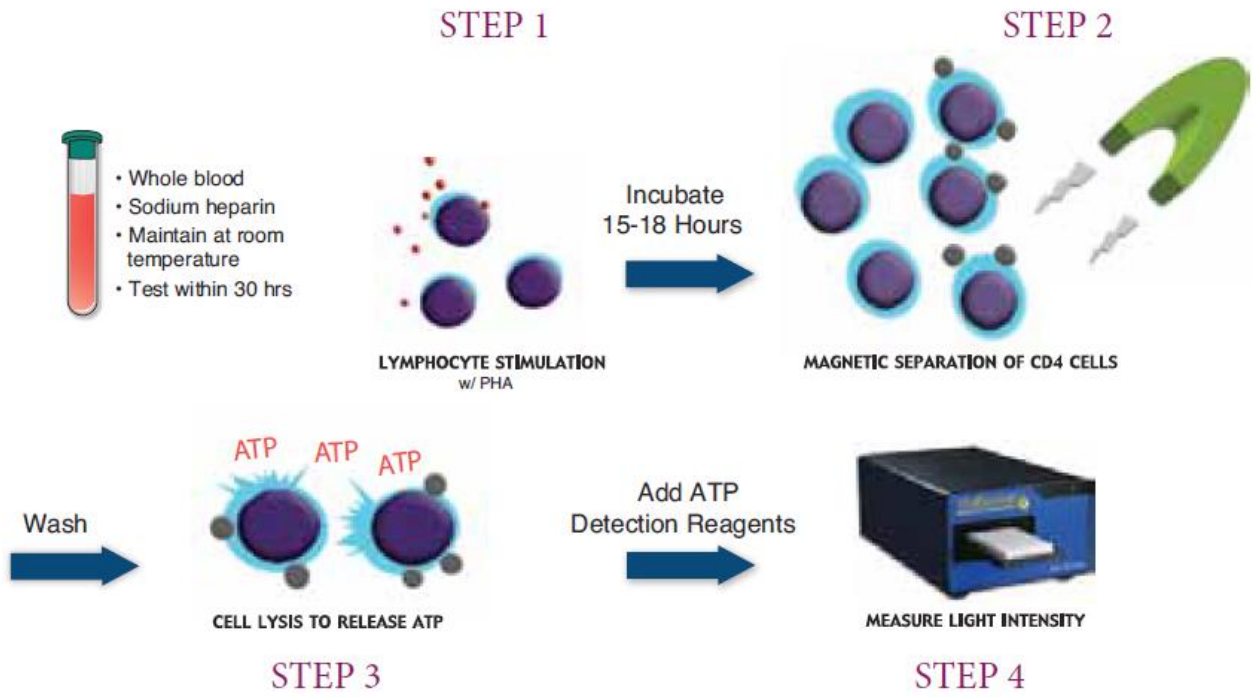

Figure 6. Technical Methodology of the Cylex ImmuKnow Assay. A schematic diagram describing the laboratory protocol of the Cylex ImmuKnow assay. (modified by Gesundheit et al 2010)

Cell isolation from BAL and from peripheral blood

Macrophages were isolated from BAL of patients with hemoptysis sine causa by adhesion procedure. Briefly, 5 x 10^6 cells/well were seeded in 24-well plates in RPMI medium for 2 h at 37°C to allow adhesion.

For neutrophil isolation, a buffy coat sample from peripheral blood of donors was separated by gradient stratification with lympholyte (Cedarlane Laboratories, Burlington, ON, Canada). CD3 + T cells were purified from peripheral blood of donors by negative magnetic beads separation (CD3 + T-cell Isolation Kit, Miltenyi Biotec, Gergisch Gladbach, Germany).

Cells treatment with GNP

Macrophages were treated for 2 h with 25 µg/ml of GNP-HCe or GNP-HC and with GNP-PEGe or GNP-PEG. The effect of nanoparticles was assayed after 48 h incubation by evaluating viability, IL-8 release and ROS production in both unstimulated and macrophages stimulated by adding 0.01 mg/ml lipopolysaccharide (LPS, Sigma-Aldrich, Milan, Italy). Long-time effect on macrophages

viability and activity was also assessed at 7 and 10 d after nanoparticle treatment. Neutrophils were treated for 4 h with GNP-HC, GNP-HCe, GNP-PEG and GNP-PEGe. Activation was assayed by evaluating the elastase release, ROS production and apoptosis. Un-functionalized nanoparticles with or without drug inside, GNP-PEGe and GNP-PEG, respectively, were also tested on macrophages and neutrophils to assay possible stimulating action of the nanoparticles per se. CD3 + T lymphocytes were treated with 25 µg/ml of GNP-HC and GNP-HCe. The effect of the nanoparticles on CD3 + T cells was evaluated by assaying proliferation rate, apoptosis and the release of IFN-g, IL-17 and IL-10 both in unstimulated and lymphocytes stimulated by adding 0.15 mg/ml phytohemagglutinin (PHA). As controls, untreated cells and cells treated with everolimus (0.003 µg/ml) in the same experimental conditions used for nanoparticles treatment were used, as previously described (Cova et al., 2015). Long-time effect of nanoparticle incubation on lymphocytes viability was also assessed at 7 and 10 d after treatment. For these experiments, only PHA-treated cells were considered since it is widely known that unstimulated lymphocyte survival is limited to 48 h. Uptake of GNP-HC and GNP-PEG labeled with AF488 by macrophages, neutrophils and lymphocytes was evaluated after 2 h incubation at 37° C in the complete medium by flow cytometry.

IL-8, IFN-g, IL-17, IL-10 and elastase detection

IL-8 production by unstimulated or stimulated (LPS treatment for 24 h) macrophages was evaluated by ELISA. The activation of neutrophils was assayed by the elastase release (enzymatic assay). Briefly, 2 x 10^7 isolated neutrophils were treated with 5 µg/ml cytochalasin B (Sigma-Aldrich, Milan, Italy) for 10 min at room temperature. Afterwards, cells were seeded in 96-well plate and treated for 20 min at 37° C with different stimuli (nanoparticles or N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 10^{-4} M), as a positive control). After centrifugation (1000 g for 10 min at 4°C) supernatants were added to a substrate solution (N-succinyl-AlaAla-Ala-p-nitroanilide, 1 mm in phosphate buffer), incubated for 1 h at 37°C and read at 405 nm spectrophotometer beam. The data were expressed as % vs. untreated neutrophils. IFN-g detection was evaluated at 24 h after incubation while IL-10 and IL-17 dosage was performed after 48 h. IFN-g, IL-17 and IL-10 production was evaluated by ELISPOT procedure, as previously described (Bianco et al., 2005).

Cell proliferation, viability and apoptosis evaluation

Proliferation and apoptosis rate were evaluated by carboxyfluorescein succinimidyl ester (BD Pharmingen, Milan, Italy) and Annexin V (Molecular Probes, Life Technologies, Milan, Italy) by flow cytometry. Lymphocyte proliferation and apoptosis were evaluated at 24, 48 and 72 h while only apoptosis was considered for long time experiments (7 and 10 d after treatments). Neutrophil apoptosis was assayed after 4 h incubation. Proliferation and apoptosis were expressed as a percentage of dividing and apoptotic cells, respectively. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test (Sigma-Aldrich, Milan, Italy) was used to assay macrophage viability at 24, 48, 72 h and 7 and 10 d incubation and was expressed as a percentage of variation vs. untreated cells.

Reactive oxygen species (ROS) detection

ROS level was detected at t0 (baseline) and after 4 h of incubation with nanoparticles for neutrophils or 48 h for macrophages by electron paramagnetic resonance (EPR) spectroscopy (Bruker, Germany) operating at the common X-Band microwave frequency (9.8 GHz). For ROS trapping, culture medium and cells were incubated with 1 mM CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) probe prepared in Krebs-Hepes buffer containing 25 lM deferoxamine methanesulfonate salt chelating agent and 5 lM sodium diethyldithiocarbamate trihydrate at pH 7.4 (Faoro et al., 2011). Spectra were recorded and analyzed by using a standard software (Win EPR version 2.11). Neutrophils and macrophages were re-suspended in 250 ll of CMH probe (1:1) and incubated for 30 min at 37 C. Afterwards, samples were frozen at 77K before acquisition by EPR (Mrakic-Sposta et al., 2012).

Animal experiments for aerosol treatment

This study was conducted under EU guidelines for the care and use of laboratory animals in accordance with Italian and European legislation (D.lgs. 116/92, European Directives 86/609/EE for the protection of animals used in scientific and experimental studies and 2010-63UE) and was approved by Ethical Committee recognized from University of Pavia. Twenty-two C57/BL6 mice were purchased from Charles River (Charles River laboratories, Lecco, Italy) and housed in polycarbonate cages for 10 d after arrival. Inhaled mice were treated in a home-made chamber in which nanoparticles in suspension were administrated by a commercial aerosol machine. Mice were divided into four groups: (1) three mice living in normal conditions for 3 d and 4 mice living in

normal conditions for 2 weeks; (2) four mice subjected to aerosol treatment for 3 d, 30 min/day, with 50 lg/mouse of GNP-HCe; (3) eight mice subjected to aerosol treatment for 2 weeks, 5 d/week, 30 min/day, with 50 lg/mouse of GNP-HCe (4 mice) or GNP-HC (4 mice); (4) three mice subjected to an aerosol treatment for 2 weeks, 5 d/week, 30 min/day, with 50 µg/mouse of GNP-HC marked with IR-820 dye. For inhalation, nanoparticles were suspended in phosphate buffer. Immediately after the last inhalation, mice were anesthetized with intraperitoneal injection of 0.5 mg/g of Avertin (2, 2, 2-Tribromoethanol) and were sacrificed by cervical dislocation. The dose of nanoparticles administered to mice by inhalation (50 µg/mice/30 min inhalation) was chosen according to the in vitro data in which we selected 25 µg/ml as the more effective concentration and taking in account mice lung volume as in table 3.

Parameter	**Value**
Aerosol concentration (ng/cm^3), experimentally set	50
Inhalation period (min), experimentally set	30
Tidal volume (cm^3), from literature[1,2]	0,18
Breathing frequency (1/min), from literature[2,3]	165
Nanoparticles (µg/min), experimentally set	1,5
Inhaled volume (cm^3/30 min), calculated from data above	891
Nanoparticles inhaled (µg/ml), experimentally set	44,5
Diameter of Au NP (nm, Mean ± SD), experimentally measured	46,8 ± 2,9

Table 3. parameter used for GMP inhalation experiment on mice (Cova et al 2017)

Bronchoalveolar lavage and IL-8 dosage

Third group and control mice were subjected to BAL after anesthetization and before sacrifice to measure IL-8 production by ELISA and to determine differential cell count by optical microscopy. BAL fluid was collected by using two consecutive instillations of PBS (1 ml) at room temperature

and was centrifuged at 1500 rpm at 4 C for 10 min; supernatants were collected and stored at -80 °C for measurement of IL-8 levels. Cell pellets were re-suspended in 100 ll PBS and cytospin samples stained with Papanicolaou dye for the differential cell count that were expressed as a percentage of macrophages, neutrophils and lymphocytes.

Histological examination of mice tissues

Paraffin-embedded sections of 5 µm of lungs, spleen, kidneys and liver isolated from control mice or mice treated for 2 weeks with GNP-HC and GNP-HCe were stained with hematoxylin and eosin for evaluation. For lungs, the degree of alveolar congestion, hemorrhage, leukocyte infiltration and the thickness of the alveolar wall were assessed. For liver, we examined the presence of alterations in the architecture, in the hepatocytes, in portal triads and sinusoids, inflammation and degeneration, Kupffer cells hyperplasia, necrosis, fatty impairment and portal fibrosis. For kidneys, we observed proximal and distal tubules, glomerular network, the volume of capsules in renal corpuscle, inflammation and fibrosis. For spleen, the degree of congestion of red pulp, inflammation, fibrosis and widening sinusoids were evaluated.

Near infrared light microscopy (NIR) technology

After sacrifice, mice treated for 2 weeks with IR820 dye-NH2 labeled GNP-HC were perfused with cold PBS and then with 4% paraformaldehyde solution. Lungs, liver, spleen and kidneys were removed and left for 24 h in paraformaldehyde. For relative quantification of signal intensity, images were obtained using the OdysseyVR Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Briefly, isolated organs were placed on the surface of the image and high-resolution scans were performed with the following parameters: near-infrared 700 and near-infrared 800 scan intensity 3, resolution 21 lm, and focus 2.5 mm. For quantitative evaluation, five regions of interest were traced in the organs, and near-infrared 700 signal intensity values were recorded using OdysseyVR software.

Transmission electron microscopy

Samples obtained from lungs of mice treated for 3 d or 2 weeks with GNP-HC or GNP-HCe were fixed by 2.5% glutaraldehyde in PBS. After three washes with the same buffer, the samples were postfixed for 2 h with 1.5% osmium tetroxide in PBS, dehydrated by a rising alcohol scale and included in epoxin resin. Ultrathin slices (70/80 nm) were cut and dyed with uranyl acetate and

citrate lead.

Animal experiments for inhalation treatment

This study was conducted at Columbia University in New York, USA. This study was conducted under US guidelines for the care and use of laboratory animals in accordance with and was approved by Ethical Committee recognized from Columbia University. The studies proposed are in vivo on sprague Dawley rats (7 weeks old/ 250g). They were anesthetized in a chamber with 4-5% isoflurane. The rats were then intubated endotracheally and ventilated for several minutes with the same dose of isoflurane, to assure deep sleep.

Ventilation and Nebulization: Harvard VentElite was connected in series with the anesthesia machine and the AeroNeb vibrating mesh Lab animal nebulizer. (figure 7). We then introduced our gnp diluted in saline into the VIBRATING MESH nebulizer and only at this point was the isoflurane was reduced to 3% and eventually completely completely cut-off for the rat to be awaken, extubated and placed in its cage to recover. After 24 hours, the rats were re-anesthetized with 4-5% isoflurane and CT scanned using the Quantum FX micro-CT scanner, equipped with internal anesthesia nose-cone, to obtain coronal images of the lungs as well as 3d renderings

Post-scan the rats were euthanized, their organs (lungs, kidneys, spleen and liver) procured and embedded in OCT for storage. The tissue treated with FITC-GNPs was fluorescently scanned with Aperio Image Scope to confirm the presence of nanoparticles.

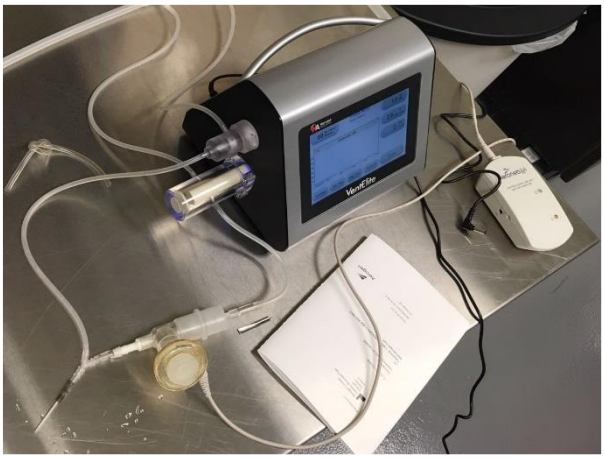

Figure 7. Harvard VentElite connected in series with anesthesia machine and AeroNeb vibrating mesh Lab animal nebulizer

Statistical analysis

The performed statistical analysis were specific for any single paper depending on the purposes of the study (Coiffard et al 2018, Cova et al 2017, Piloni et al 2016, Piloni et al 2017, Ruttens et al 2017).

RESULTS

Aims 1: to find and study unknown risk factor which may have an impact on chronic rejection development and survival after lung transplantation.

In order to identify potential unknown risk factor during the first year of this PhD, 13 lung transplant centers, including IRCCS Policlinico San Matteo Foundation, in 10 European countries created a cohort of 5707 patients to quantify the association between air pollution and development of CLAD and survival (Ruttens et al, 2017).

The patients characteristics are shown in table 4.

Center	Newcastle	Oslo	Pavia	Utrecht	Vienna	Zurich
N-value	546	234	220	195	624	275
Time of lung transplantation	1987-2011	1990-2011	1991-2011	1989-2011	1989-2011	1992-2011
Age mean (SD)	47 (13)	51 (11)	46 (13)	46 (13)	49 (13)	43.8 (16)
Gender(male) n-value (%)	278 (51)	112 (48)	149 (68)	96 (49)	330 (53)	144 (52)
Double lung transplantation, n-value (%)	353 (65)	188 (80)	132 (60)	152 (78)	483 (77)	259 (94)
Underlying disease n-value (%)						
Emphysema	150 (27)	142 (61)	38 (17)	85 (44)	360 (58)	68 (25)
Cystic fibrosis	201 (36)	18 (8)	42 (19)	59 (30)	88 (14)	100 (36)
Interstitial lung disease	102 (19)	24 (10)	70 (32)	34 (17)	107 (18)	64 (23)
Pulmonary arterial hypertension	41 (8)	15 (6)	45 (21)	4 (2)	44 (7)	16 (6)
Other	52 (10)	35 (15)	25 (11)	13 (7)	25 (4)	27 (10)
Mean (SD) road length (m) in a specific buffer zone						
50m buffer zone	96 (48)	234 (49)	127 (68)	116 (58)	88 (59)	94 (62)
100m buffer zone	331 (141)	119 (162)	464 (197)	462 (173)	330 (166)	356 (167)
200m buffer zone	1218 (467)	479 (555)	1563 (660)	1708 (536)	1174 (495)	1303 (553)
500m buffer zone	6498 (2604)	2763 (2830)	7915 (3492)	9621 (2934)	6374 (2854)	7057 (2856)
1000m buffer zone	22164 (9557)	9888 (9493)	26352 (12982)	33729 (11068)	22423 (11532)	23317 (8922)
PM_{10} mean (SD)	19 (3)	13 (2)	33 (7)	27 (2)	24 (4)	/
Distance to freeway mean (SD)	29113 (47812)	50387 (77672)	11716 (15059)	4959 (5232)	10912 (15411)	5436 (7075)
Distance major road mean (SD)	2862 (4058)	1493 (2238)	3959 (6177)	2272 (2728)	2036 (3109)	1595 (2048)

Center	Barcelona	Copenhagen	Essen	Groningen	Hannover	Leuven	Munich
N-value	400	533	192	406	1087	583	412
Time of lung transplantation	1997-2011	1992-2011	1999-2011	1990-2011	1987-2011	1991-2011	1990-2011
Age mean (SD)	46 (15)	48 (12)	52 (13)	44 (14)	44 (13)	48 (14)	47 (13)
Gender(male) n-value (%)	230 (58)	247 (46)	109 (57)	196 (48)	587 (54)	309 (53)	195 (47)
Double lung transplantation, n-value (%)	285 (71)	232 (43)	180 (94)	325 (80)	938 (86)	439 (75)	245 (59)
Underlying disease n-value (%)							
Emphysema	151 (38)	351 (66)	95 (51)	173 (43)	328 (30)	270 (46)	119 (29)
Cystic fibrosis	63 (16)	76 (14)	33 (17)	100 (25)	250 (23)	94 (16)	57 (14)
Interstitial lung disease	130 (33)	59 (11)	47 (25)	59 (15)	229 (21)	121 (21)	193 (47)
Pulmonary arterial hypertension	18 (5)	28 (5)	5 (3)	43 (11)	115 (11)	50 (9)	14 (3)
Other	38 (10)	19 (4)	9 (5)	31 (8)	165 (15)	48 (8)	29 (7)
Mean (SD) road length (meter) in a specific buffer zone							
50m buffer zone	179 (73)	71 (57)	93 (50)	105 (58)	85 (52)	100 (49)	93 (59)
100m buffer zone	639 (225)	272 (153)	330 (133)	399 (164)	315 (132)	311 (125)	356 (145)
200m buffer zone	2400 (636)	1046 (460)	1237 (442)	1554 (578)	1148 (423)	1017 (463)	1277 (462)
500m buffer zone	13122 (3337)	5791 (2524)	6707 (2368)	8367 (3321)	6099 (2190)	5268 (2535)	6628 (2507)
1000m buffer zone	45375 (14002)	20109 (9552)	23383 (8177)	28893 (12517)	20294 (7932)	18146 (8874)	21615 (9171)
PM_{10} mean (SD)	32 (5)	19 (2)	25 (3)	25 (3)	22 (2)	26 (2)	22 (2)
Distance to freeway mean (SD)	9455 (21120)	16624 (30116)	5051 (6249)	7832 (8435)	12647 (14167)	7025 (6222)	12410 (12074)
Distance major road mean (SD)	2671 (4857)	5785 (7559)	1477 (1601)	3055 (3962)	1716 (2550)	2073 (2280)	2264 (2979)

Table 4. Patient characteristics and exposures per lung transplant center (from Ruttens et al 2017, supplementary material)

Of these 5707 patients, 2626 (46.0%) developed CLAD and 2577 patients (45.2%) died.

Median time to CLAD was 6.4 years, while median survival was 8.9 years; however, patients with early death were excluded from this analysis, which will significantly impact survival. Age, sex, underlying disease and type of transplant were available for all subjects.

Concentrations of air pollutants varied between and within study centres (figure 8).

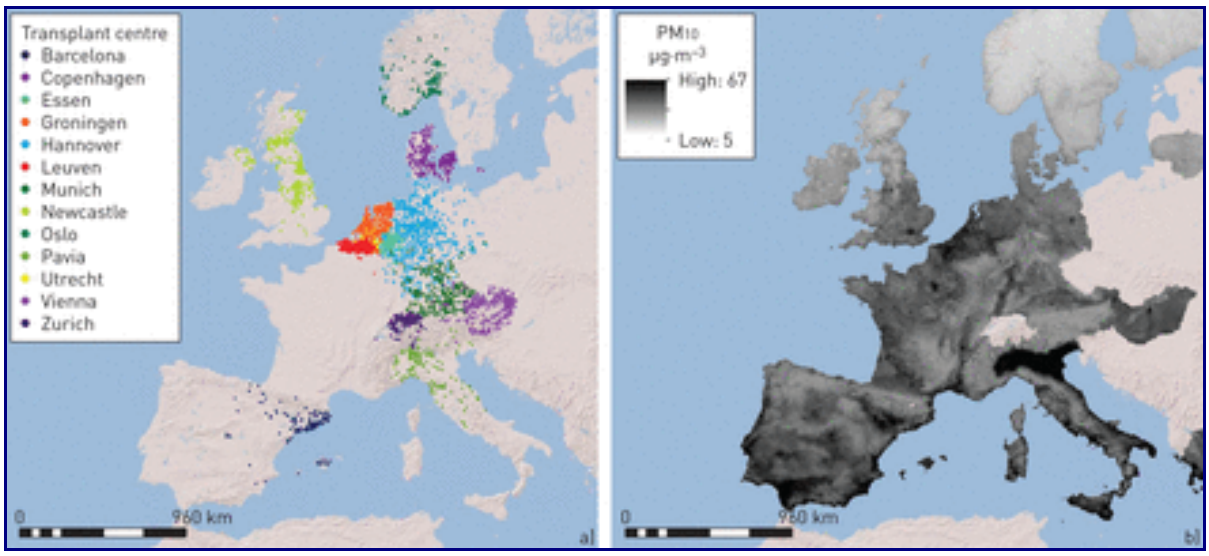

Figure 8. a) Geographical distribution of the lung transplant patients from the 13 different lung transplant centres in 10 different European countries. Each point represents a single patient. b) Average particulate matter with aerodynamic diameter ≤10 µm (PM10) concentration in Western Europe. No PM10 values were available for Zurich (From Ruttens et al 2017)

An association of all-cause mortality with road length in the 100, 200 and 500 m buffer zones and a trend for the 50 m buffer zone were observed. Figure 9 a and b shows the HR values for mortality and road length in a 200 m buffer zone and PM10 for each center. We could not detect an association between CLAD and PM10, road length in 50 and 100 m buffer zones, and distance to a major road or freeway, although PM10 tended to be significant (figure 9 d).

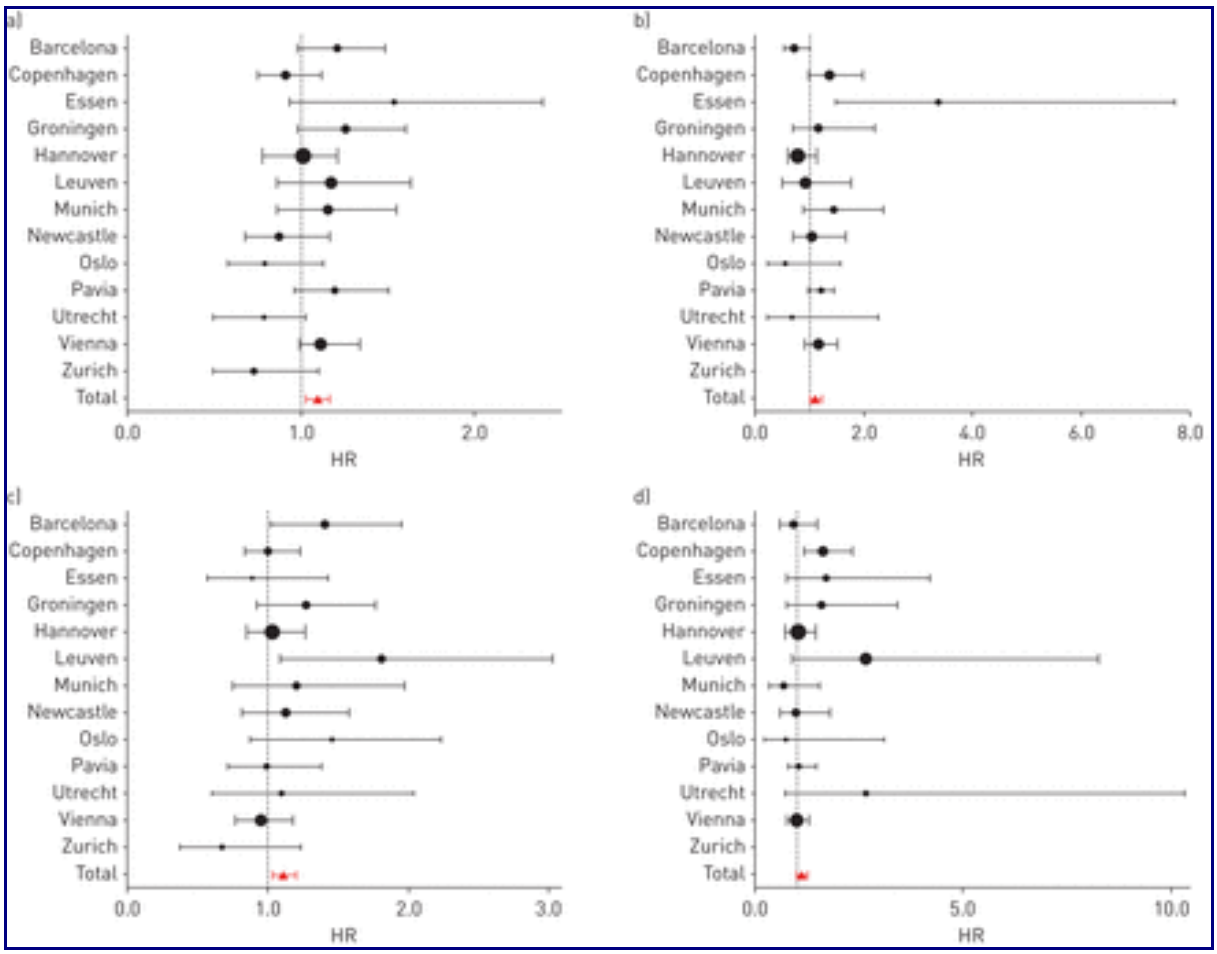

Figure 9. Hazard ratio (HR) values for a) all-cause mortality and road length within a 200 m buffer zone around the patient's home address, b) all-cause mortality and particulate matter with aerodynamic diameter ≤10 µm (PM10), c) CLAD and road length in a 200 m buffer region, and d) CLAD and PM10. Each HR is per interquartile range increase. The HR values shown are from the macrolide-free group and are corrected for date of lung transplantation, type of transplantation, underlying disease, sex and age. No PM10 values were available for Zurich. The size of the signal reflects the number of subjects and the lines represent the 95% confidence interval. (From Ruttens et al 2017)

Based on previous literature on the protective effect of azithromycin on air pollution (Nawrot et al, 2011). We considered *a priori* that macrolide therapy could modify the relation between air pollution and outcomes, and we therefore divided the patients in two groups, according to whether they had ever (2151 patients (37.7%)) or never (3556 patients (62.3%)) been chronically treated with azithromycin or clarithromycin (Table 5).

	Macrolide-free group	Macrolide group
Patients	3556	2151
Recipient age years	46.5±14	45.5±13.8
Male	1836 (52)	1146 (53)
Underlying disease		
Emphysema	1535 (43)	798 (37)
Cystic fibrosis	707 (20)	474 (22)
Interstitial lung fibrosis	737 (21)	502 (23)
Pulmonary arterial hypertension	274 (8)	164 (8)
Other	303 (9)	213 (10)
Type of transplantation		
Double or heart–lung	2559 (72)	1652 (77)
Single	997 (28)	499 (23)
Year of transplantation		
1987–1995	479 (14)	94 (4)
1996–2000	710 (20)	254 (12)
2001–2005	936 (26)	644 (30)
2006–2011	1431 (40)	1159 (54)
CLAD		
No	2185 (62)	891 (40)
Yes	1367 (39)	1259 (60)
Unknown	5 (0)	1 (0)
Death or graft loss	1937 (55)	640 (30)

Table 5. Characteristics of the lung transplant cohort divided into patients taking macrolides or not. (From Ruttens et al 2017)

The deleterious effect of air pollution in macrolide users is absent in patients taking macrolides before CLAD or not developing CLAD, while a trend towards an association is observed in patients who started with macrolide therapy at/after CLAD diagnosis (*i.e.* no protective effect on CLAD development was possible as diagnosis was made at/after initiation and therefore macrolides were started "too late"). (Table 6)

	IQR	Macrolide-free group		Macrolide group	
		HR (95% CI)	p-value	HR (95% CI)	p-value
Death[#]					
Road length in buffer zone					
50 m	108 m	1.055 (0.955–1.112)	0.076	0.989 (0.824–1.047)	0.23
100 m	279 m	1.111 (1.025–1.202)	0.0094[+]	1.003 (0.875–1.150)	0.95
200 m	752 m	1.094 (1.030–1.779)	0.0054[+]	0.978 (0.872–1.094)	0.66
500 m	4092 m	1.085 (1.000–1.130)	0.0356[+]	1.085 (0.960–1.226)	0.15
1000 m	15403 m	1.047 (0.985–1.131)	0.12	1.080 (0.970–1.202)	0.17
PM_{10}	6 $\mu g \cdot m^{-3}$	1.081 (1.000–1.167)	0.049[+]	0.982 (0.859–1.120)	0.77
Distance to freeway	1233 m	0.987 (0.964–1.012)	0.16	1.000 (0.883–1.025)	0.31
Distance to major road	241 m	1.000 (0.976–1.024)	0.68	0.976 (0.907–1.000)	0.092
CLAD[¶]					
Road length in buffer zone					
50 m	108 m	1.025 (0.956–1.099)	0.49	0.997 (0.927–1.073)	0.93
100 m	279 m	1.076 (0.975–1.190)	0.14	0.943 (0.848–1.048)	0.28
200 m	752 m	1.110 (1.023–1.204)	0.0114[+]	0.949 (0.872–1.030)	0.21
500 m	4092 m	1.130 (1.042–1.226)	0.0010[+]	0.960 (0.884–1.085)	0.62
1000 m	15403 m	1.113 (1.031–1.202)	0.0115[+]	0.970 (0.897–1.063)	0.48
PM_{10}	6 $\mu g \cdot m^{-3}$	1.093 (0.988–1.208)	0.076	0.886 (0.803–0.976)	0.013[+]
Distance to freeway	1233 m	0.988 (0.964–1.012)	0.26	1.012 (1.000–1.038)	0.13
Distance to major road	241 m	1.000 (0.976–1.024)	0.73	1.024 (0.976–1.049)	0.44

IQR: interquartile range; HR: hazard ratio; PM_{10}: particulate matter with aerodynamic diameter ⩽10 µm. [#]: macrolide-free group n=3556, macrolide group n=2151; [¶]: macrolide-free group n=3551, macrolide group n=2150; [+]: statistically significant (p<0.05). All parameters were analysed for every IQR increase. In the Cox analysis we corrected for patient age, patient sex, native disease (chronic obstructive pulmonary disease *versus* interstitial lung disease *versus* cystic fibrosis and bronchiectasis *versus* pulmonary hypertension *versus* others), type of transplantation (single *versus* sequential single) and date of transplantation (1987–1995 *versus* 1996–2000 *versus* 2001–2005 *versus* 2006–2011).

Table 6. Overview of the main results investigating the association of particulate air pollution and traffic exposure with mortality and chronic lung allograft dysfunction (CLAD) in lung transplant patients. IQR: interquartile range; HR: hazard ratio; PM10: particulate matter with aerodynamic diameter ≤10 µm. #: macrolide-free group n=3556, macrolide group n=2151; ¶: macrolide-free group n=3551, macrolide group n=2150; +: statistically significant (p<0.05). All parameters were analysed for every IQR increase. In the Cox analysis we corrected for patient age, patient sex, native disease (chronic obstructive pulmonary disease versus interstitial lung disease versus cystic fibrosis and bronchiectasis versus pulmonary hypertension versus others), type of transplantation (single versus sequential single) and date of transplantation (1987–1995 versus 1996–2000 versus 2001–2005 versus 2006–2011).

Another unexplored risk factor has been assessed during a 4-month research fellowship at the Lung Transplant Unit at Columbia University of New York: the role of pathological findings in donor lungs and their association with the long term outcome of lung transplant recipients.

A retrospective analysis of 102 consecutive lung allograft pathology reports of transplanted lungs undergone downsizing by non-anatomical lung resections at the time of transplant was performed.

Survival, chronic lung allograft dysfunction (CLAD) development, pulmonary function, bronchoalveolar infection and acute rejection were assessed.

Pathological findings were reported in 64% (65/102) of donor lungs: 22/65 (34%) had bronchial abnormalities (bronchiolitis, inflammation, broncho-pneumonia or granulomas); 38/65 (58%) had alveolar abnormalities (emphysema); 15/65 (23%) showed vascular abnormalities (single or multiple thromboemboli). (Figure 10)

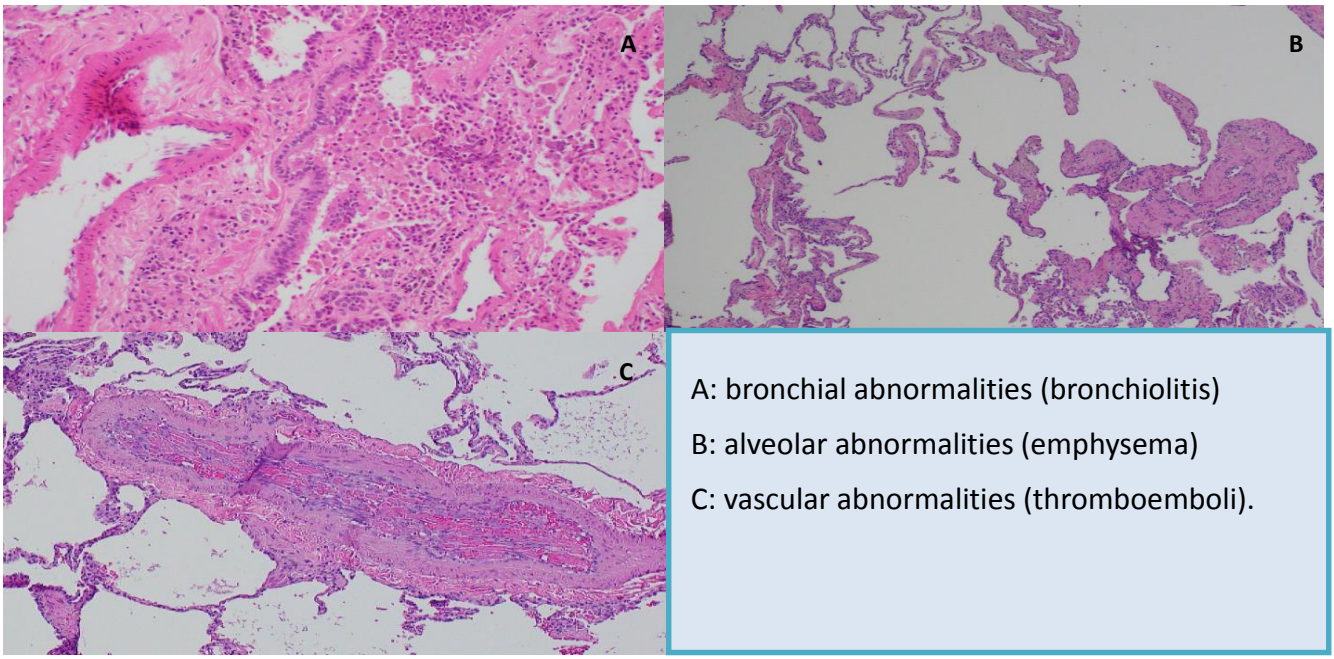

Figure 10. Hystopathological findings.

No differences were found regarding infections rate, acute rejections episodes or best-FEV1 after transplant (Table 7)

	no Findings (N 37)	all Findings (N 65)	Bronchial alt. (N 22)	Alveolar alt. (N 38)	Vascular alt. (N 15)
SURVIVAL (months, median)	57,8	56	57,05	56	56,4
CLAD-FREE (months, median)	43,95	42,8	43,95	42,88	42,53
Episodes of Acute Rejection (N, mean)	1,43	1,4	1,43	1,41	1,4
Episodes of Infections (N, mean)	5,94	5,74	5,87	5,74	5,84
Best-FEV1 (L, mean)	2,29	2,3	2,3	2,3	2,28

Table 7.

The non-adjusted Kaplan-Meyer survival curves showed no difference in terms of survival or CLAD development between all groups (Figure 11)

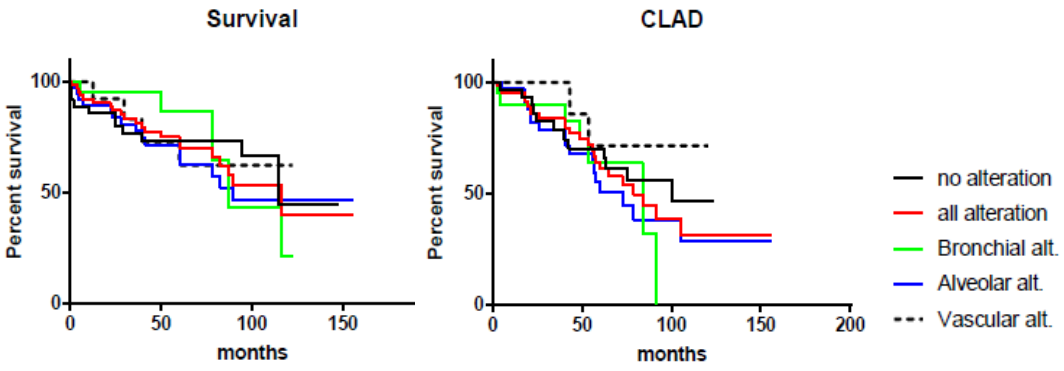

Figure 11. Kaplan-Meyer curves showing no differences between the groups.

Aims 2: to explore the immunological risk factors of BOS onset and development apart from the occurrence of Acute Rejections.

In a first work (Piloni et al, 2017) we have decided to analyze the role of Regulatory T cell ($CD4^{+}CD25^{high}CD127^{-}$ T-reg) in the pathogenesis of BOS confirming the possible role of T-regulatory cells in the onset and evolution of BOS. The role of $CD4^{+}CD25^{high}CD127^{-}$ T-reg cells in Tx acceptance has been studied in experimental models and also in clinics. Animal studies demonstrated that these cells are expanded in tolerized animals. These data have been partially confirmed in kidney and liver Tx recipients, showing a positive correlation between graft survival and the number of circulating $CD4+CD25^{+}$ T-reg cells, as well as a correlation between their peripheral fluctuation and the occurrence of acute and chronic rejection. As for lung Tx, evidence is limited and somewhat contradictory. To date, however, little is known about the long term evolution of peripheral $CD4^{+}CD25^{high}CD127^{-}$ regulatory T cells in lung Tx .

Patients

Overall 137 patients were included in this retrospective study and followed-up for a median of 105.9 months (6.7-310.5). Demographics and clinical features of included patients are listed in Table 8, including gender, age at Tx, Tx indication, type of Tx, length of follow-up and type of immunosuppressive drugs used. Some patients (27%) were enrolled in the immunological follow- up at time of Tx, while the others entered the study later in the FU period (median FU months at first determination in the latter group: 82,4 months, range 14,0–275,7) this , as stated above, was considered in the statistical analysis. Being a prospective immunological FU, the overall number of included samples is high : n° 1943 with a median of 14 sample/patient (range 2-39).

	N° of Patients = 137
Mean age at Tx (years ± SD)	46.1 ± 12.86
Sex (M:F)	91:46
Lenght of post-Tx follow-up (months/range, median)	105.9 / 6.7-310.5
Tx Indications	
Emphysema / Alpha1 antitrypsin Deficiency	27
Primary graft disfunction	4
Bronchiectasis / Cystic Fibrosis	26
Interstitial lung disease	52
Pulmonary hypertension / Ebstein's disease / Eisenmenger Syndrome / Mounier-Kuhn Syndrome	25
Rare pulmonary conditions	3
Type of Tx	
Single lung Tx	46
Double lung Tx	83
Heart and Lung Tx	8
Immunosuppression therapy	
Cyclosporine	23
Tacrolimus	119
Azathioprine	15
Mycophenolate mofetil	88
Rapamycin	37
Prednisone	137
Extracorporeal photopheresis (ever)	39
Azitromycin (ever)	98

Table 8. demographic and clinical features of the patients enrolled in this study. (Piloni et al 2017)

Variables associated to T-reg cell counts

Results of bivariate and multivariate analysis are shown in Table 9. As for acute rejection, the limited number of samples obtained during an episode of acute cellular (A grade ≥2, any B grade) or humoral rejection did not allow us to assess any statistical association with peripheral T-reg cell number**.** All tested immunological variables (CD3+ ,CD4+, CD8+, CD19+ and CD16+CD56+ cells) resulted significantly associated to T-reg in a positive relation (Table ….) at the initial bivariate analysis. Among clinical variables only the presence of CLAD, treatment with azathioprine and ECP were significantly associated to peripheral $CD4^{+}CD25^{high}CD127^{-}$ T-reg cell counts, while

azithromycin, kidney failure, infections of any type and neoplasia did not show any influence. $CD4^{+}CD25^{high}CD127^{-}$ T-reg cell counts resulted slightly but significantly higher in patients undergoing treatment with azathioprine (p=0.03) with respect to other immunosuppressive regimens. On the other hand, the presence of CLAD, either BOS (p≤0.01) or RAS (p≤0.01) and treatment with ECP (P≤ 0.001) were associated to a significant decrease in T-reg cell counts. At multivariate analysis, only the association with azathioprine was lost (Table 9).

		Bivariate			**Multivariate**		
Variable	**Category**	**Coef**	**95% CI**	**P-value**	**Coef**	**95% CI**	**P-value**
Time	Per each month since Tx	0.16	0.10 to 0.22	**<0.001**	0.18	0.12 to 0.24	**<0.001**
CLAD	RAS	-6.60	-11.594 to-1.603	**0.01**	-5.19	-10.2 to -0.16	**0.04**
	BOS	-8.14	-11.807 to -4.473	**<0.001**	-6.51	-10.26 to -2.77	**0.001**
BOS grade	0p	-2.05	-4.165 to 0.069	0.06			
	1	-2.53	-5.122 to 0.068	0.06			
	2	-6.49	-9.724 to -3.261	**<0.001**			
	3	-4.90	-8.731 to -1.066	**0.01**			
Lymphocytic population	cd3	0.009	0.008 to 0.01	**<0.001**			
	cd3cd4	0.021	0.019 to 0.023	**<0.001**			
	cd3cd8	0.01	0.008 to 0.011	**<0.001**			
	cd19	0.05	0.038 to 0.057	**<0.001**			
	cd16cd56	0.01	0.005 to 0.008	**<0.001**			
	cd4cd25high	0.60	0.585 to 0.62	**<0.001**			
Immunosuppresive therapy	Cyclosporine	0.20	-3.598 to 4.007	0.92			
	Tacrolimus	-2.71	-5.783 to 0.367	0.08			
	Azathioprine	4.65	0.438 to 8.856	**0.03**	-2.12	-6.73 to 2.48	0.336
	Mycophenolate	0.04	-1.735 to 1.823	0.96			
	Rapamycin	-0.75	-3.202 to 1.703	0.55			
	Prednisone	-6.32	-15.329 to 2.687	0.17			
Azithromycin therapy		-0.74	-2.505 to 1.016	0.41	0.58	-1.28 to 2.43	0.542
Extracorporeal photopheresis		-6.03	-8.259 to -3.804	**<0.001**	-5.65	-8.05 to -3.25	**<0.001**
Kidney failure		-1.36	-3.62 to 0.907	0.24			

Table 9. bi- and multi-variate linear regression analysis per $CD4^{+}CD25^{high}CD127^{-}$ Linear mixed models were fitted, with patient and time since Tx as random effects, and individual predictors

and time as fixed effects. CLAD-Chronic lung allograft dysfunction, RAS-Restrictive allograft syndrome, BOS-Bronchiolitis obliterans syndrome. (Piloni et al 2017)

Furthermore, when BOS severity grade was considered, a significant progression of $CD4^{+}CD25^{high}CD127^{-}$ T-reg cell decline was observed (Figure 12).

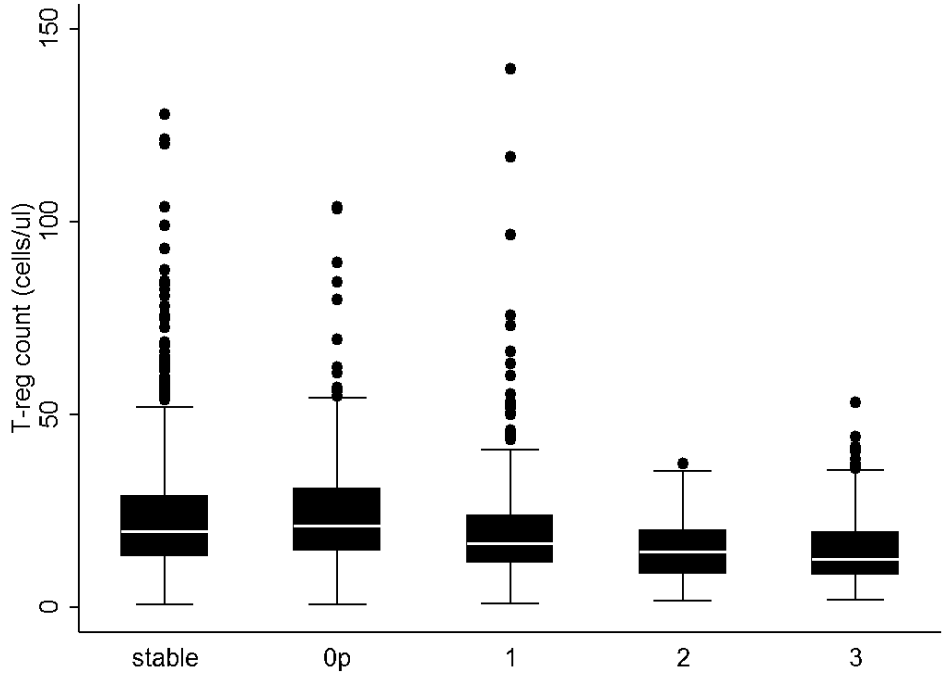

Figure 12. Relation between T-reg cells count and BOS grade (calculated according to BOS severity classification). (Piloni et al 2017)

Prediction of CLAD

In Table 10, we show that in patients with higher mean peripheral $CD4^{+}CD25^{high}CD127^{-}$ T-reg counts, the risk of presenting CLAD or progressing in the graft dysfunction in the subsequent trimester was significantly lower (OR 0.97, 95%CI 0.95-0.99, p=0.032).

CLAD grade	OR	P-value	95% Conf. interval
CD4+CD25highCD127-	0.97	0.012	0.95 to 0.99
Azithromycin therapy	5.80	<0.001	2.81 to 11.99
Prednisone	0.22	<0.001	0.13 to 0.39
Trimester	1.01	<0.001	1.01 to 1.02

Table 10. ordered logistic regression analysis for association between CLAD grade and a number of predictors, including T-reg in the previous trimester. in ordered logistic regression models, OR>1

implies higher risk of being in a higher category (in this case: CLAD grade, calculated for all CLAD patients according to BOS severity classification) rather than in any of the lower categories; an OR <1, implies a lower risk.

In a second paper, submitted by Piloni et al, 2018, we investigate the role of B-regulatory cells (CD19+CD24highCD38high B cells), given the great interest in the transplantation community, even if the informations about Breg cells are scarce. Only one work by Chong and coll. showed an higher level of Breg cells (transitional Bcell) but not of memory B cells in the peripheral blood of patients with stable graft function after kidney transplantation (Chong et al 2011), thus suggesting a protective role of this cell subtype in graft tolerance, confirmed later by Silva and coll.

Patients

Overall, 117 patients were included in this retrospective study and followed-up for 108.7±66.4 months (6.7-310.5 months from transplant). Demographics and clinical features of included patients are listed in Table 11 including gender, age at transplant, transplant indication, type of transplant, length of follow-up and type of immunosuppressive drugs used.

N° of Patients = 117	
Mean age at Tx (years ± SD)	45.8 ± 12.5
Sex (M:F)	74:43
Lenght of post-Tx follow-up (months/range, media)	108.7 ± 66.4 / 6.7-310.5
Tx Indications	
Emphysema / Alpha1 antitrypsin Deficiency	21
Primary graft disfunction	1
Bronchiectasis / Cystic Fibrosis	25
Interstitial lung disease	45
Pulmonary hypertension / Ebstein's disease / Eisenmenger Syndrome / Mounier-Kuhn Syndrome	23
Rare pulmonary conditions	3
Type of Tx	
Single lung Tx	38
Double lung Tx	71
Heart and Lung Tx	8
Total number of determination = 1106	
Immunosuppression therapy	
Cyclosporine	134
Tacrolimus	948
Azathioprine	21
Mycophenolate mofetil	551
Rapamycin	230
Prednisone	1106
Extracorporeal photopheresis	697
Azithromycin	317

Table 11. demographic and clinical features of the patients enrolled in this study.

Being a retrospective immunological FU, the overall number of included samples is high: n° 1106 with a median of 11 samples/patient (IQR 8-15).

Functional Characterization of $CD19^{+}CD24^{high}CD38^{high}$ B cells

On 5 samples of representative blood samples from lung transplant recipients, we showed that the 96,4% of $CD19^{+}CD24^{high}CD38^{high}$ B cell subset is IL-10 producing cells. (Figure 13).

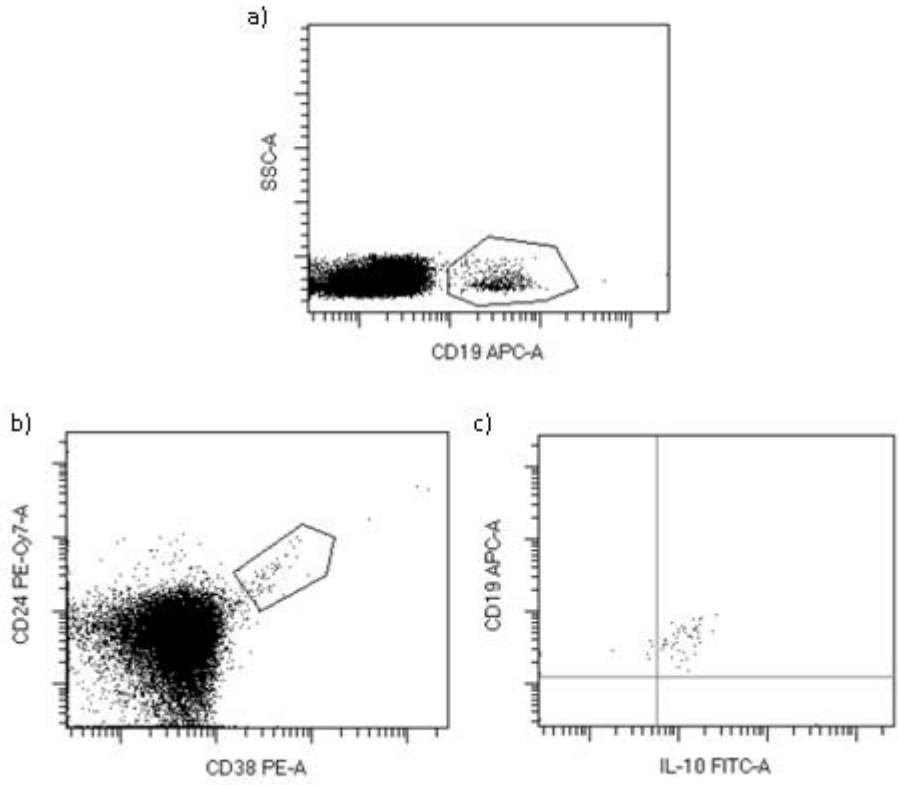

Figure 13. Representative flow cytometry analysis of Breg cells IL-10 producing in isolated PBMCs. a) Lymphocytes were gated according to forward and site scatter, then CD19 cells were analyzed; b)C D19 cells were analyzed for CD24 and CD38; c) CD19CD24^{high}CD38^{high} IL-10^{+} B cells.

Variables associated to B-reg cell counts

Results of bivariate and multivariate analysis are shown in Table 12.

All tested immunological variables (CD3+ ,CD4+, CD8+, CD19+, CD16+CD56+ and CD25high cells) resulted positively associated to B-reg (Table 2) at the initial bivariate analysis. As for clinical variables, only five were significantly associated to CD19+CD24highCD38high B-reg cells count: the presence of chronic rejection (p=0.036), treatment with azathioprine (p=0.041) and mycophenolate mofetil (p<0.001), pulmonary infections such as *Staph. Aureus* (p=0.005) and *A. fumigatus* (p=0.003). Of note, we did not find any significant association with a specific phenotype of chronic rejection (BOS or RAS) or with BOS severity, as well as no association with acute rejection episodes, although

a low number of episodes in our cohort, was found. At multivariate analysis, all but one (azathioprine treatment) associations found in the univariate analysis were confirmed (Table 12).

		Bivariate			Multivariate		
Variable	**Category**	**Coef**	**95% CI**	**P-value**	**Coef**	**95% CI**	**P-value**
CLAD	Ever	-0,19	-0.37 to -0.01	**0,036**	-0.21	-0.39 to -0.03	**0.022**
	RAS	-0,44	-0.95 to 0.06	0,086			
	BOS	-0,07	-0.42 to 0.28	0,699			
BOS grade	0p	-0,20	-.399 to .009	0,061			
	1	-0,14	-.379 to .094	0,237			
	2	-0,18	-.495 to .137	0,266			
	3	-0,31	-.674 to .056	0,097			
Lymphocytic population	cd3	0	0 to 0	0			
	cd3cd4	0,001	0 to .001	0			
	cd3cd8	0	0 to 0	0,001			
	cd19	0,004	.003 to .005	0			
	cd16cd56	0	0 to 0	0,002			
	cd4cd25high	0,002	0 to .005	0,071			
Immunosuppresive therapy	Cyclosporine	-0,16	-.598 to.27	0,459			
	Tacrolimus	0,03	-.261 to .328	0,823			
	Azathioprine	0,63	.025 to 1.238	**0,041**	0.54	-0.06 to 1.14	0.076
	Mycophenolate	-0,38	-.549 to -.215	**<0.001**	-0.36	-0.53 to 0.20	**<0.001**
	Rapamycin	-0,003	-.244 to .239	0,984			
	Prednisone	-0,38	-.984 to .204	0,198			
Azithromycin therapy		-0,11	-.289 to .068	0,226			
Extracorporeal photopheresis		0,07	-.157 to .298	0,546			
Kidney failure		-0,06	-.287 to .155	0,557			
Infections							
	Bacterial	0,11	-.085 to .322	0,254			
	Staph aureus	0,61	.183 to 1.051	**0,005**	0.63	0.20 to 1.01	**0.004**
	Viral	-0,05	-.207 to .094	0,464			
	Fungal	0,13	-.161 to .418	0,385			
	Aspergillus spp	0,58	.199 to .962	**0,003**	0.51	0.14 to 0.88	**0.007**

Table 12. bi- and multi-variate linear regression analysis for CD19+CD24highCD38high Breg. linear mixed models were fitted, with patient and time since Tx as random effects, and individual predictors and time as fixed effects. CLAD-Chronic lung allograft dysfunction, RAS-Restrictive allograft syndrome, BOS-Bronchiolitis obliterans syndrome.

B-reg and T-reg correlation

Since literature data suggest that a possible interaction between B and T regulatory cells "in vivo" might bring to an expansion of T regulatory clones, we aimed to assess whether a correlation between B and T cell counts was detectable in lung transplant recipients. As shown in figure 14, we could not detect any statistically significant association between CD19+CD24highCD38high B-reg cells and $CD4^{+}CD25^{high}CD127^{-}$ T-reg cells counts.

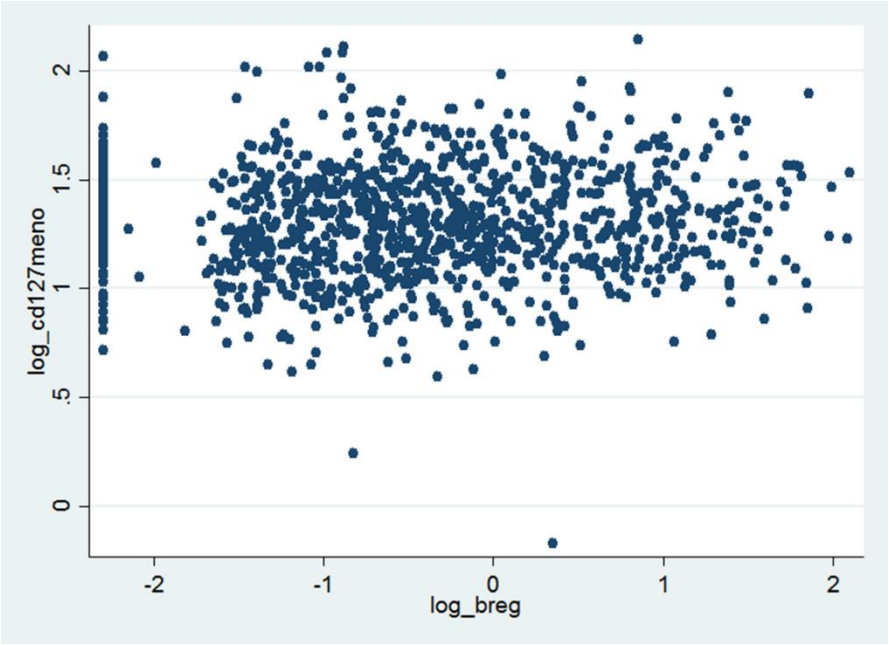

Figure 14. Scatterplot of T-reg cells counts and B-reg cells counts showing no correlation between cells population

Immunosuppressive therapy is the cornerstone of preventing allograft rejection in solid organ transplantation, thus, we have analyzed the effect of different immunosuppressive strategies, either as induction or as manteinance regimen, on T cell reactivity and transplant outcome.

In a paper, patients from 3 different lung transplant centers in Europe who encountered different induction strategies (no induction, anti-thymocyte globulins and basiliximab) were compared in terms of circulating lymphocyte kinetics and the possible impact of these latter on the short-term allograft outcome (infection, acute rejection and mortality) after lung transplantation. (Coiffard, Piloni et al, 2018).

Patient characteristics

164 recipients were included in the study, 50 from Pavia (Italy) who did not receive any T-cells specific induction therapy, 57 from Strasbourg treated with basiliximab and 57 from Marseille who were treated with ATG. Recipients from Pavia had greater weights in pre-Tx (median 72 kg [IQR, 57-80]) compared to Marseille and Strasbourg (58 kg [50-74] and 59 kg [48-72], respectively, p=0.004). Pulmonary fibrosis was more represented in Pavia (39%) and Marseille (40.4%) and emphysema in Strasbourg (47.4%), p<0.001. There were no significant between-group differences in one-year outcome: pulmonary infection (76.1%), acute rejection (39.0%) and one-year mortality (12.8%). In pre-Tx, blood lymphocyte count of the study population was 2000 cells/µL [1510-2540] (median [IQR]). Blood lymphocytes were the lowest (495 cells/µL [238-900]) at day 7 and then demonstrated a slow and constant increase until day 730 but never returning to pre-Tx values (1640 cells/µL [1185-2180] at day 730, p<0.001 compared to pre-Tx) (Table 13).

	Total (n=164)	Pavia (n=50)	Marseille (n=57)	Strasbourg (n=57)	p
Induction		Steroids	ATG	Basiliximab	
Age	50 [39-59]	51 [41-58]	48 [38-56]	55 [38-60]	0.35
Sex (men)	107 (65.2)	39 (78.0)	32 (56.1)	36 (63.2)	0.056
Weight (before LTx)	61 [51-76]	72 [57-80]	58 [50-74]	59 [48-72]	0.004
Disease					
Cystic Fibrosis	32 (19.5)	2 (4.0)	14 (24.6)	16 (28.1)	<0.001
Emphysema	60 (36.6)	13 (26.0)	20 (35.1)	27 (47.4)	
Pulmonary Fibrosis	64 (39.0)	29 (58.0)	23 (40.4)	12 (21.1)	
Bronchiectasis	3 (1.8)	3 (6.0)	0 (0.0)	0 (0.0)	
PH	5 (3.0)	3 (7.0)	0 (0.0)	2 (3.5)	
Lymphocytes					
Before LTx	2000 [1510-2540]	1600 [1166-2075]	2240 [1590-2540]	2145 [1867-2695]	<0.001
Day 7	495 [238-900]	1100 [800-1475]	234 [122-481]	430 [240-602]	<0.001
Day 14	788 [500-1205]	1192 [747-1469]	650 [432-940]	650 [382-1015]	<0.001
Day 30	965 [531-1480]	1204 [868-1630]	1012 [627-1603]	620 [430-1102]	<0.001
Day 90	1306 [810-1860]	1628 [1270-2027]	1400 [817-1962]	930 [645-1365]	<0.001
Day 180	1339 [932-1880]	1691 [1283-2139]	1450 [943-1865]	1080 [710-1360]	<0.001
Day 365	1490 [1116-1898]	1717 [1430-2233]	1530 [1145-1899]	1160 [862-1497]	<0.001
Day 730	1640 [1185-2180]	1789 [1457-2518]	1620 [1207-2320]	930 [890-1835]	0.002
1 year outcome					
Infection	124 (76.1)	38 (76.0)	45 (80.4)	41 (71.9)	0.58
Acute rejection	64 (39.0)	22 (44.0)	17 (29.8)	25 (43.9)	0.21
Mortality	21 (12.8)	4 (8.0)	11 (19.3)	6 (10.5)	0.18

Table 13. Patient's characteristics according to the lung transplant center. Results are expressed as median [interquartile] or number, n (%). Statistical analysis was performed using Chi2 or Kruskal-

Wallis tests as appropriate. Quantitative values were not normal distributed (Shapiro-Wilk test). ATG= anti-thymocyte globulins, LTx= lung transplantation, PH= pulmonary hypertension. (Coiffard et al 2018)

Effect of induction therapy

Multivariate analysis demonstrated significant differences according to the centers and lymphocyte counts in pre-Tx. Compared to recipients from Pavia, lymphocyte suppression in recipients from Marseille and Strasbourg was higher, +914 cells/µL [+647 to +1181], p<0.001 and +923 cells/µL [+644 to +1201], p<0.001 respectively. Recipients with lymphocytes higher than the median of the study population had higher lymphocyte suppression compared to the others, +1079 (+1287 to +871), p<0.001 (Table 14).

Analysis	Univariate		Multivariate	
	beta (95% CI)	p	beta (95% CI)	p
Center				
Pavia		**reference**		
Marseille	**1303 (994 to 1611)**	**<0.001**	**914 (647 to 1181)**	**<0.001**
Strasbourg	**1322 (1013 to 1630)**	**<0.001**	**923 (644 to 1201)**	**<0.001**
Age (/year)	-9 (-21 to 2)	0.11	-3 (-14 to 7)	0.52
Sex (Men)	-258 (-584 to 78)	0.12	-1 (-248 to 247)	0.99
Pre-Tx weight (/kg)	-13 (-23 to -3)	0.01	-2 (-10 to -7)	0.67
Pre-Tx Ly level (High)	**1358 (1588 to 1129)**	**<0.001**	**1079 (1287 to 871)**	**<0.001**
Disease				
Cystic Fibrosis		reference		
Emphysema	-137 (-700 to -427)	0.96		
Pulmonary Fibrosis	-687 (-1248 to -127)	0.008	37 (-328 to 404)	0.83
Bronchiectasis	-929 (-2484 to 626)	0.46		
PH	-1204 (-2443 to 34)	0.06	-264 (-448 to 976)	0.46
1-year outcome				
Infection	-213 (-582 to 155)	0.25		
Acute rejection	-183 (-502 to 135)	0.26		
Death	411 (-70 to 892)	0.09	208 (-130 to 546)	0.23

Table 14. Blood lymphocyte suppression at day 7 according to the induction therapy, recipient's characteristics and allograft outcome. Results expressed the estimation (beta) of the difference (with confidence interval) between groups of the lymphocyte count suppressions, which corresponds to the difference between the value in pre-transplantation and day 7. "Pre-Tx Ly level" is a variable which separates equally the population in 2 groups according to the pre-transplant lymphocyte level. Univariate analysis was performed using analysis of variance (ANOVA) and multivariate analysis using the linear model and confirmed by bootstrapped ANOVA for variables with p value<0.25 in univariate analysis. CI= confidence interval, LTx= lung transplantation, Ly=lymphocytes, PH= pulmonary hypertension. (Coiffard et al 2018)

Lymphocyte reconstitution

We then analysed lymphocyte reconstitution on all repeated measures from day 7 to day 730. There was at each time-point an increase of lymphocyte level when compared to day 7 (p<0.001). Multivariate analysis demonstrated significant differences according to centers, weight and lymphocyte counts in pre-Tx, lung disease and one-year mortality (Table 15 and Figure 15). Recipients from Marseille and Strasbourg had lower lymphocytes compared to Pavia, -625 cells/µL (-779 to -471), p<0.001 and -641 cells/µL (-800 to -481), p<0.001, respectively. There was however no difference between Marseille and Strasbourg. Whereas weight had no influence on lymphocytes in pre-Tx, weight was associated after transplantation with higher lymphocytes +10 cells/µL/kg (+5 to +15), p<0.001. This positive correlation was significantly present at day 7, 14 and 30 (Figure 16). Lymphocytes were higher in recipients with lymphocytes higher to the median in pre-Tx, +243 cells/µL (+124 to +362), p<0.001. Emphysema was associated with lower lymphocytes compared to cystic fibrosis, -228 cells/µL (-445 to -10), p=0.04. There was no difference for other aetiologies. One-year mortality was associated with lower lymphocytes -324 cells/µL (-519 to -127), p<0.001.

Analysis	Univariate		Multivariate	
	beta (95% CI)	p	beta (95% CI)	p
Lymphocytes				
Day 7		**reference**		**reference**
Day 14	**258 (163 to 352)**	**<0.001**	**369 (22 to715)**	**0.04**
Day 30	**434 (322 to 545)**	**<0.001**	**861 (456 to 1266)**	**<0.001**
Day 90	**701 (590 to 811)**	**<0.001**	**1114 (724 to 1503)**	**<0.001**
Day 180	**725 (622 to 828)**	**<0.001**	**1033 (662 to 1404)**	**<0.001**
Day 365	**913 (769 to 1058)**	**<0.001**	**952 (437 to 1467)**	**<0.001**
Day 730	**1078 (930 to 1227)**	**<0.001**	**1257 (727 to 1787)**	**<0.001**
Center				
Pavia		**reference**		**reference**
Marseille	**-649 (-806 to -491)**	**<0.001**	**-625 (-779 to -471)**	**<0.001**
Strasbourg	**-615 (-772 to -458)**	**<0.001**	**-641 (-800 to -481)**	**<0.001**
Age (/year)	-3 (-9 to 2)	0.26		
Sex (Men)	117 (-44 to 278)	0.15	-95 (-239 to 49)	0.20
Pre-Tx weight (/kg)	**10 (5 to 15)**	**<0.001**	**10 (5 to 15)**	**<0.001**
Pre Tx Ly level (High)	100 (-54 to 254)	0.20	**243 (124 to 362)**	**<0.001**
Disease				
Cystic Fibrosis		reference		reference
Emphysema	-131 (-341 to 79)	0.22	**-228 (-445 to -10)**	**0.04**
Pulmonary Fibrosis	130 (-78 to 338)	0.22	-224 (-675 to 226)	0.33
Bronchiectasis	435 (-144 to 1014)	0.14	-160 (-372 to 52)	0.14
PH	-79 (-540 to 382)	0.74	-201 (-623 to 204)	0.32
1-year outcome				
Infection	9 (-173 to 190)	0.92		
Acute rejection	92 (-66 to 249)	0.25		
Mortality	**-346 (-579 to -112)**	**0.004**	**-324 (-519 to -127)**	**0.001**

Table 15. Blood lymphocyte reconstitution according to the induction therapy, recipient's characteristics and allograft outcome. Results expressed the estimation (beta) of the lymphocyte count differences (with confidence interval) between groups. "Pre-Tx Ly level" is a variable which separates equally the population in 2 groups according to the pre-transplant lymphocyte level. Analysis of repeated measures (lymphocyte counts from day 7 to day 370) was performed with the linear mixed model. Multivariate was performed for variables with univariate p-values <0.25. (Coiffard et al 2018)

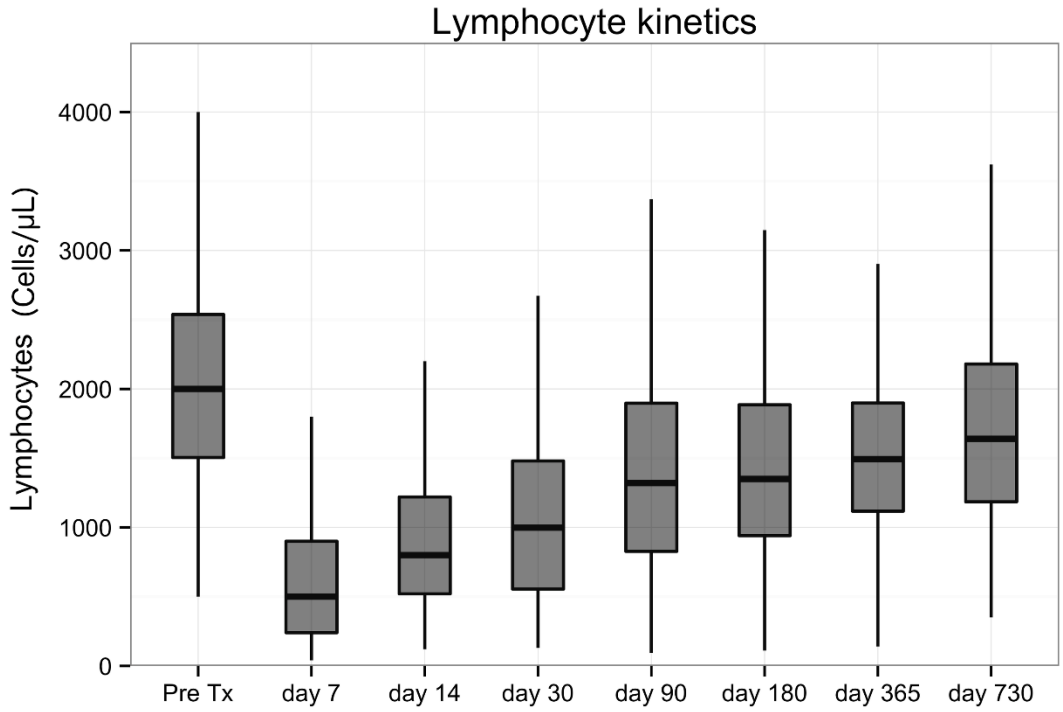

Figure 15. Kinetics of blood lymphocyte counts over the study period. Y-axis= lymphocytes counts in cells/µL and X-axis= time post-transplant in days. The boxplots correspond to the medians with inter-quartile range (distance between the first and third quartiles); the lower and upper whiskers extend from the hinge to the lowest and highest (respectively) values that is within 1.5xIQR of the hinge. Tx= transplantation (Coiffard et al 2018)

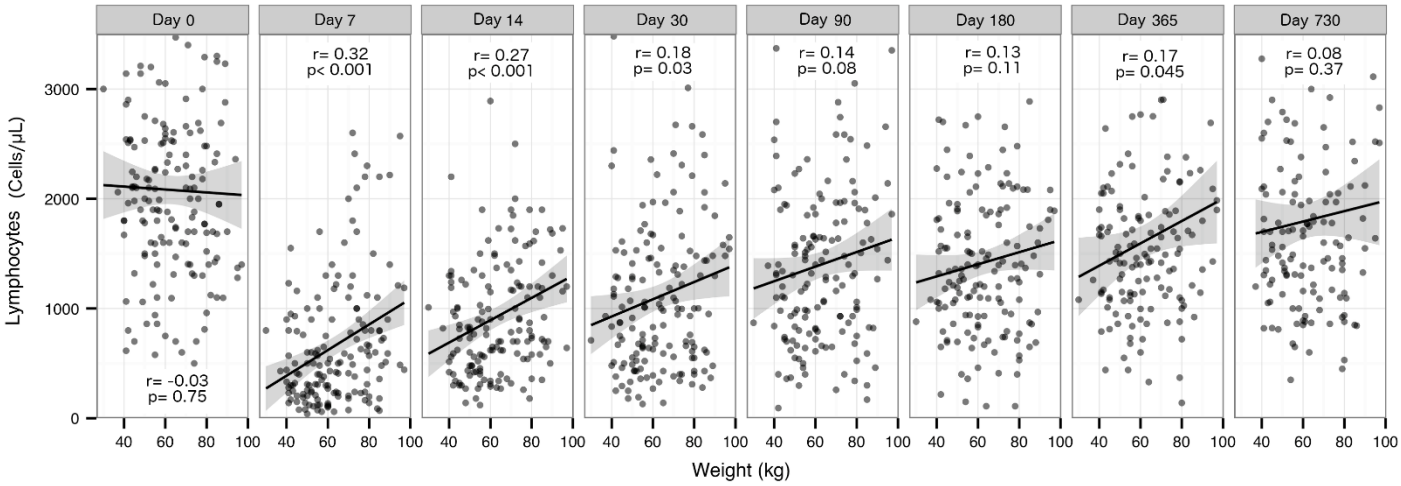

Figure 16. Scatterplots of blood lymphocyte counts over the study period according to the pre-transplant recipients' weight. Y-axis= lymphocytes counts in cells/µL and X-axis= recipients' weight in kilogramme. Dots represent each recipient included. Lines correspond to the fitted linear regression of the lymphocyte counts with confidence interval. The linear dependence between weight and lymphocytes was assessed using the Pearson's correlation test. (Coiffard et al 2018)

Lymphocyte predictive value

One-year mortality was associated with lower lymphocytes in the reconstitution period. We analysed by ROC curves, at each time-points of the reconstitution (day 14, 30, 90, and 180), the diagnostic performance of lymphocytes to predict one-year mortality. The analysis revealed the best performance at day 90, AUC=0.76 (0.60 to 0.89), Se=67% and Sp=79% for a best threshold at 1000 cells/mL (Figure 17).

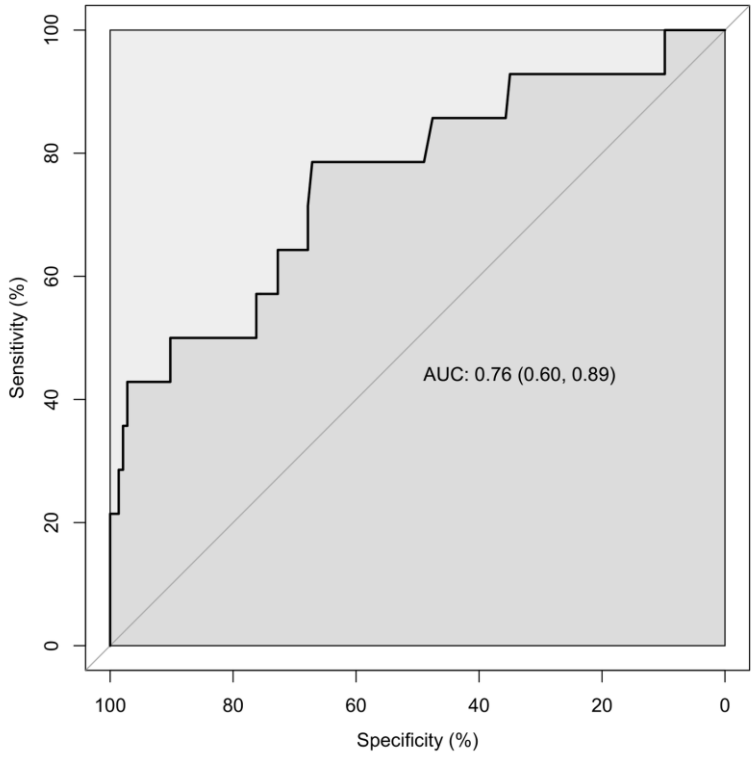

Figure 17. ROC curves of blood lymphocyte counts at day 90 for the prediction of one-year mortality. AUC: area under the curve. (Coiffard et al 2018)

As for the maintenance therapy, in another paper the burden of chronic immunosuppressive treatment was quantyfied by means of the Immuknow assay, which measures the ATP production from stimulated peripheral blood CD4+T-cells in order to analyze the rate of T cell activation and correlate it with the risk of infections and the onset of CLAD, either RAS or BOS (Piloni et al., 2016).

Patients

Our population included 47 males and 14 females. The mean age at the time of follow- up was 47 years. Underlying diseases were Idiopathic pulmonary fibrosis/NSIP (n=27), COPD/Emphysema (n=13), Pulmonary hypertension (n=6), Cystic fibrosis (n=5), Bronchiectasis (n=3), Ebstein's disease/Eisenmenger Syndromed (n=3), CLAD/RE-Tx (n=2), Combined pulmonary fibrosis and emphysema (n=1), Lymphangioleiomyomatosis (n=1). 23 patients underwent single lung transplant, 35 patients underwent double lung transplant and 3 patients underwent combined heart and lung transplant.

127 samples were collected samples from 61 patients, 71 (56%) were found to be in the over-

immunosuppression range (<226 ng/ml).

The average ImmunoKnow assay level in the over-immunosuppression group was 112.92 ng/ml (SD ± 58.2), while for the second group it was 406.14 ng/ml (SD ± 167.7).

Univariate and multivariate analysis of factors associated to ImmuKnow level are reported in Table 16.

In the over-immunosuppression group with mean IMK level ≤ 226 ng/ml, there were 51 episodes of infection among 71 serum determinations of serum (71%), for an OR of 2.754 (95%CI 1.40-5.39; *Pp*-value 0.003 at univariable analysis). Of these, 37 were bacterial, 28 viral and in 6 fungal; in the other group we found that 25 out of 56 IMK samples (44%) were taken during an infectious episode. In fact, the mean absolute ATP level was different between samples from patients with infection and samples from those without non-infection samples (202.38 ± 139.06 ng/ml *vs.* 315.51 ± 221.60 ng/ml; *Pp*<.001).

		IM level ≤226 (N = 71) Mean level = 112,92 ng/ml (SD±58.2)	IM level >226 (N = 56) Mean level = 406,14 ng/ml (SD±167)		**Univariate**			**Multivariate**	
Variable	**Category (description)**			**OR**	**95% CI**	***P*-value**	**OR**	**95% CI**	***P*-value**
Age in years	Mean	50.46	50.66	1.01	0.96-1.05	0.641			
Time from tx	Months	53.00	71.14	0.996	0.99-1.00	0.208			
Immunosuppression level	High	44	41						
	Low	27	15	1.702	0.78-3.7	0.179	1.93	0.82-4.50	0.130
WBC	Cell/ml	7109.17	8397.5						
	Lymphocyte (%)	25.46	19.85	1.052	1.01-1.09	**0.013**			
Lymphocytic subpopulations (%)	CD3+	77.7	80.5	0.977	0.94-1.01	0.231			
	CD3+CD4+	34.8	35.8	0.993	0.96-1.02	0.627			
	CD3+CD8+	39.2	41.4	0.984	0.95-1.01	0.324			
	CD3+CD19+	6.0	4.9	1.042	0.95-1.13	0.333			
	CD3+CD56+CD16+	14.8	12.9	1.02	0.98-1.59	0.328			
CLAD (76)									
	BOS (52)	28	24	1.29	0.58-2.85	0.529	1.24	0.53-2.89	0.623
	RAS (24)	21	3	9.194	2.35-35.8	**0.001**	12.16	2.86-51.62	**0.001**
Infections	Overall (single + coinfection)	51	25	2.754	1.40-5.39	**0.003**	2.82	1.42-5.62	**0.003**
	Bacterial	37	19	1.899	0.94-3.80	0.071			
	Viral	28	15	1.773	0.82-3.81	0.142			
	Fungal	6	3	0.908	0.25-3.21	0.881			

Table 16. Descriptive statistics, uni- and multi-variate logistic regression analysis of factors associated to ImmuKnow level

Interestingly, RAS was associated to IMK level, with an OR of 9.194 (95%CI 2.35-35.8, *Pp*<0.001).

Of note, the two groups did not differ in terms of low *vs.* high immunosuppressive regimen.

The area under the ROC curve of the multivariate model for IMK level ≤ 226 ng/ml was 0.768, indicating good validity of the model itself. (Figure 18)

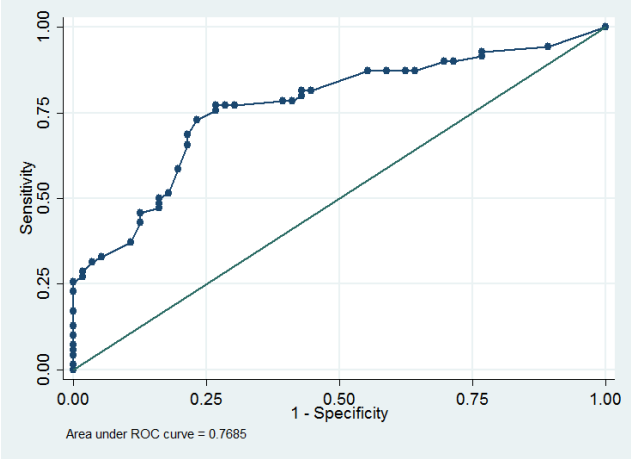

Figure 18. ROC curve

In addition, by retrospectively analyzing clinical data , we found, interestingly, found that the determination of IMK level resulted in a change in the immunosuppressive treatment (86% of the cases) by decreasing the amount of immunosuppressive drugs in the case that a low level of ATP production was detected at IMK. (Figure 19)

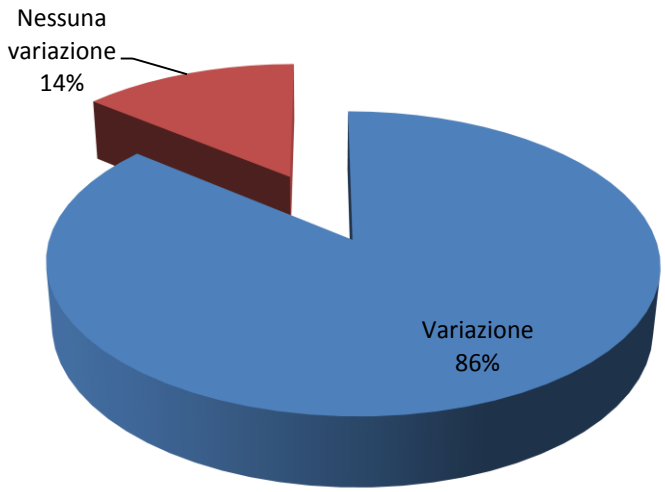

Figure 19. Percentage of subject who underwent a a change in the immunosuppressive treatment (86% of the cases) .

Aim 3: to explore the effectiveness of a newly designed therapeutic approach for BOS.

Thus systemic immunosuppressive therapy, though effective, shows a considerable load of both short and long-term side effects, responsible for the reduced survival of lung transplant recipients, the research group in Pavia designed and developed an innovative therapeutic approach of BOS, consisting in the use by inhalation of nanocarriers (Gold NanoParticles), loaded with specific anti-proliferative/immunosuppressive drugs and specifically targeted to disease cells (Fibroblasts/lymphocytes/macrophages) responsible of the pathological processes. During there years these GNPs were tested on different immune cell lines (macrophages, neutrophils and lymphocyte) to explore the inflammatory response arisen after GNPs exposure

Effect of nanoparticles on macrophage activation

Under unstimulated conditions (Figure 20), we observed that GNP-HCe significantly decreased the metabolic activity of macrophages after 72 h of treatment compared to everolimus alone and GNP-HC, suggesting that everolimus inside GNPs exerted an anti-inflammatory activity in unstimulated macrophages. Under LPS stimulation (Figure 20), GNP-HCe slightly decreased macrophage viability, but with no significant differences with respect to everolimus alone. IL-8 production by LPS-stimulated and unstimulated macrophages was not significantly changed by the treatment with all the nanoparticles used (Figure 20). Since no significant cell activation was induced by GNP-PEG and GNP-PEGe the following experiments were performed only with antibody-engineered nanovectors. The impairment of cell viability by GNP-HCe both in LPS-treated and untreated macrophages was confirmed by measurements of the mtMP, as shown by the remarkable decrease in red/green intensity ratio of JC-1 (Figure 20). As a possible sign of activation induced by nanoparticles, we also evaluated the production of ROS, as it represents a defense mechanism characteristic of phagocytes. Unstimulated cells showed a slight but significant increase in extracellular ROS production with GNP-HCe but it seemed more a release from intracellular compartment instead of a novel burst (Figure 20). On the other hand, in the case of LPS-stimulated macrophages, GNP-HCe did not stimulate intracellular and extracellular ROS production.

In our experimental conditions, the incubation with everolimus significantly increased ROS secretion in LPS-stimulated cells while GNP-HCe and GNP-HC did not. This difference could be assigned to the

presence of anti-CD44 antibody on GNP surface since an involvement of CD44 in ROS defense pathway has been documented (Ishimoto et al., 2014).

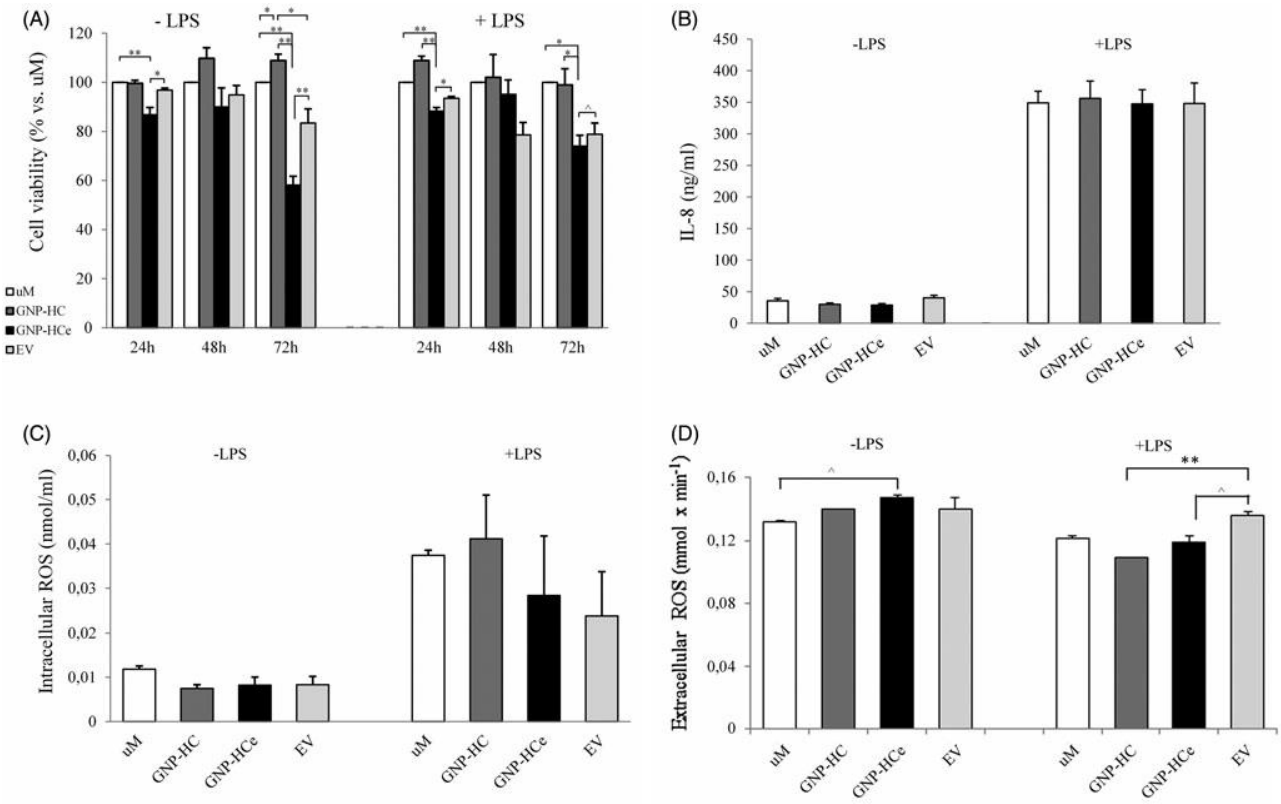

Figure 20. Effect of nanoparticles on macrophage activation. (A) Cell viability assayed at 24, 48 and 72 h after 2 h incubation. (B) IL-8 secretion by LPS-stimulated and unstimulated macrophages. (C) Intracellular and (D) extracellular ROS level evaluated at 48 h after 2 h incubation. Histograms are obtained from the means ± standard error of three experiments. ***p<0.0001; **p<0.001; *p<0.01; ^p<0.05. uM: untreated macrophages; LPS: lipopolysaccharides. (Modified from Cova et al 2017)

Effect of nanoparticles on neutrophil activation

Neutrophils have a very short half-life (6–8 h), which is even shorter when they are stimulated (Summers et al., 2010). For this reason, we could only analyze the effects of GNPHCe under basal condition as soon as they were isolated from peripheral blood, using as a positive control neutrophils stimulated with FMLP, a naturally occurring bacterial peptide that stimulates PMNs through a specific membrane receptor (Torres et al., 1993). Both GNP-HC and GNP-HCe did not affect elastase release (Figure 21). Only a slight increase in apoptotic rate was induced by GNP-HCe compared to untreated neutrophils or neutrophils incubated with GNP-HC or everolimus alone (Figure 21). GNP-HCe and everolimus alone significantly increased ROS production in

culture media with a concomitant marked a drop in intracellular free radicals suggesting a leakage of ROS from the cells rather than a burst (Figure 21).

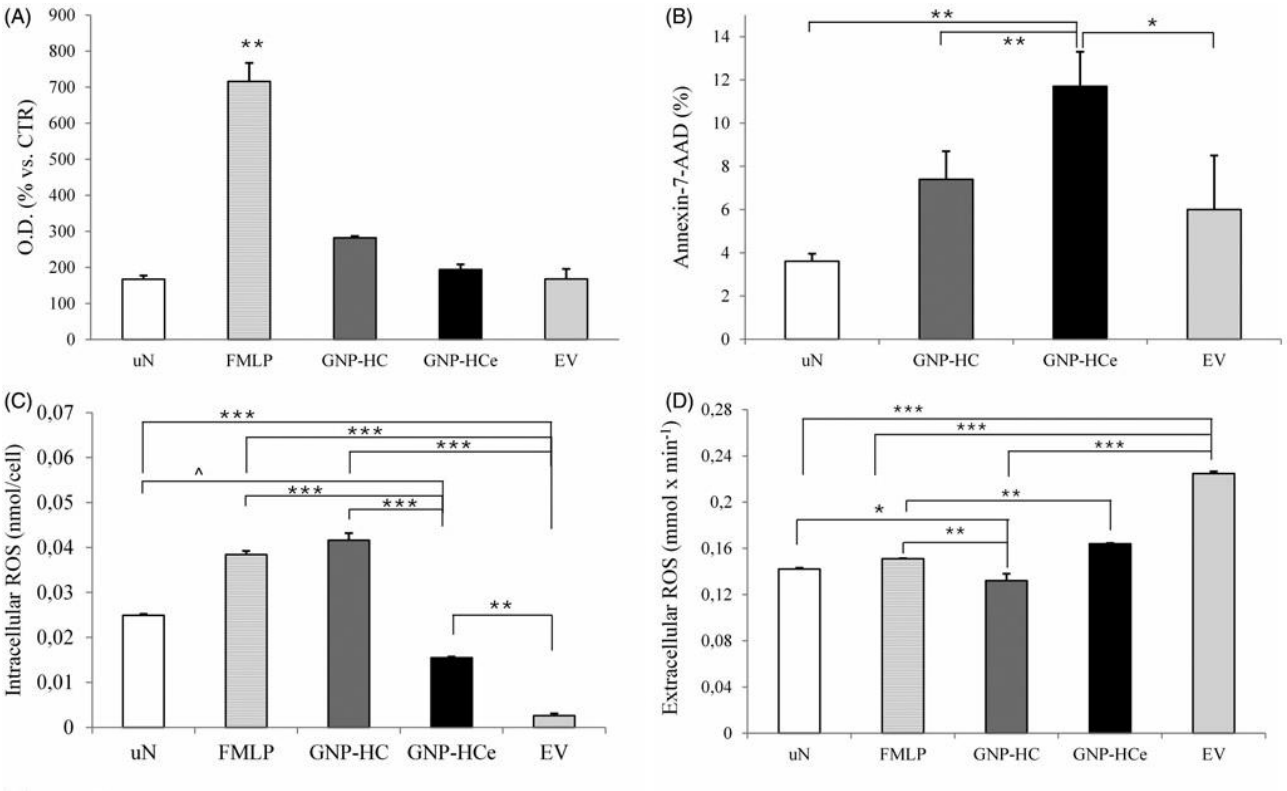

Figure 21 Effect of nanoparticles on neutrophil activation. (A) Elastase secretion. Neutrophil responsiveness was proved by elastase secretion induced by FMLP. (B) Apoptotic rate and cell death. (C) Intracellular and (D) extracellular ROS level evaluated after 2 h incubation.. Histograms are obtained from the means ± standard error of three experiments.***p<0.0001; **p<0.001; *p<0.01; ^p<0.05. uN: untreated neutrophils; FMLP: positive control. (Modified from Cova et al 2017)

Effect of nanoparticles on lymphocyte activation

Experiments were carried out with unstimulated and stimulated (PHA mitogen stimulation) T lymphocytes. Based on previous findings (Nakano et al., 2007), we decided to analyze the impact of GNP-HCe on the apoptosis rate of activated lymphocytes, as well as under basal conditions, to assess the potential of our nanoconstruct to ameliorate tolerance toward graft through the induction of T cell apoptosis. Treatment of PHA-stimulated cells with GNP-HCe resulted in significant increase in apoptotic rate after 8 h, which protracted after 24 h, as compared to everolimus alone, whose effect vanished with time (Figure 22). These results indicate a persisting action of the drug as a result of a long-lasting. The improved effect of everolimus formulated via

GNPs compared to the drug alone was particularly evident in the analysis of cytokine production by PHA-stimulated T cells since GNP-HCe significantly decreased INF-g, IL-17 and IL-10 secretion compared to everolimus alone, which, in contrast, had no effect (Figure 22).

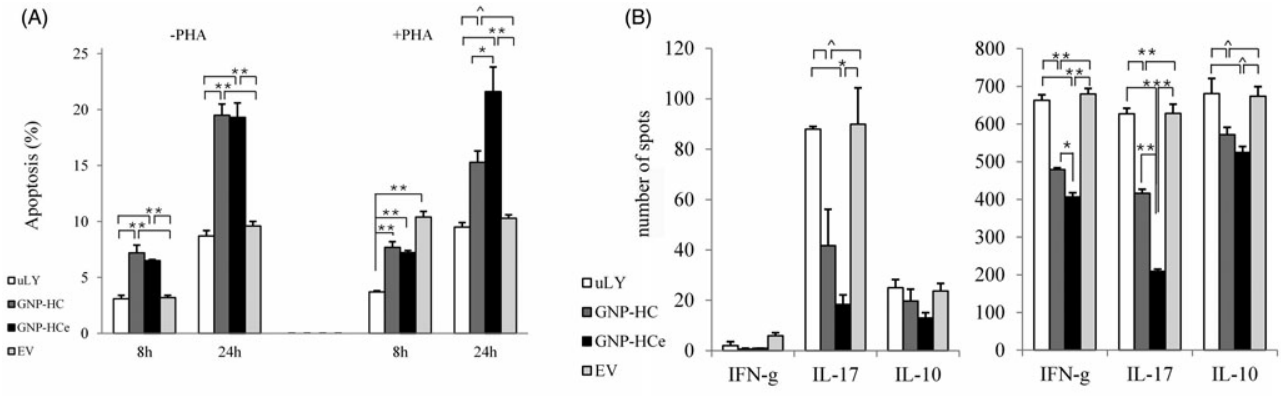

Figure 22. Effect of nanoparticles on lymphocyte activation. (A) Apoptosis evaluated at 8 and 24 h after 2 h incubation in unstimulated and PHA-stimulated lymphocytes. (B) IFN-g, IL-17 and IL-10 secretion in un-stimulated and PHA-stimulated lymphocytes. Cytokine production is expressed as a number of positive spots/1.5_105 cells for IFN-g and/3_105 cells for IL-17 and IL-10. Histograms are obtained from the means ± standard error of three experiments. ***p<0.0001; **p<0.001; *p<0.01; ^p<0.05. PHA: phytohemagglutinin. (Modified from Cova et al 2017)

Localization and effect of inhaled nanoparticles in normal mice

One of the main objectives, when we designed the nanovehicle based treatment to specifically target MCs, was to administer them by the inhalatory route. Therefore, we undertook in vivo experiments treating normal mice with: a) fluorescent-marked GNP-HC without everolimus to understand the fate of NPs and b) GNP-HCe in order to evaluate if our “fully armed” GNPs could induce an inflammatory insult. NIR technology demonstrated that mice lungs were strongly positive to fluorescence marker when inhaled with IR820-labeled GNP-HC (Figure 23) compared to untreated mice (Figure 23). Instead, no signal differences were recorded in liver, kidney and spleen between inhaled and control mice (Figure 23).

Neither significant induction of IL-8 production in BAL of mice inhaled for 2 weeks with functionalized nanoparticles with or without everolimus inside was observed(Figure 23).

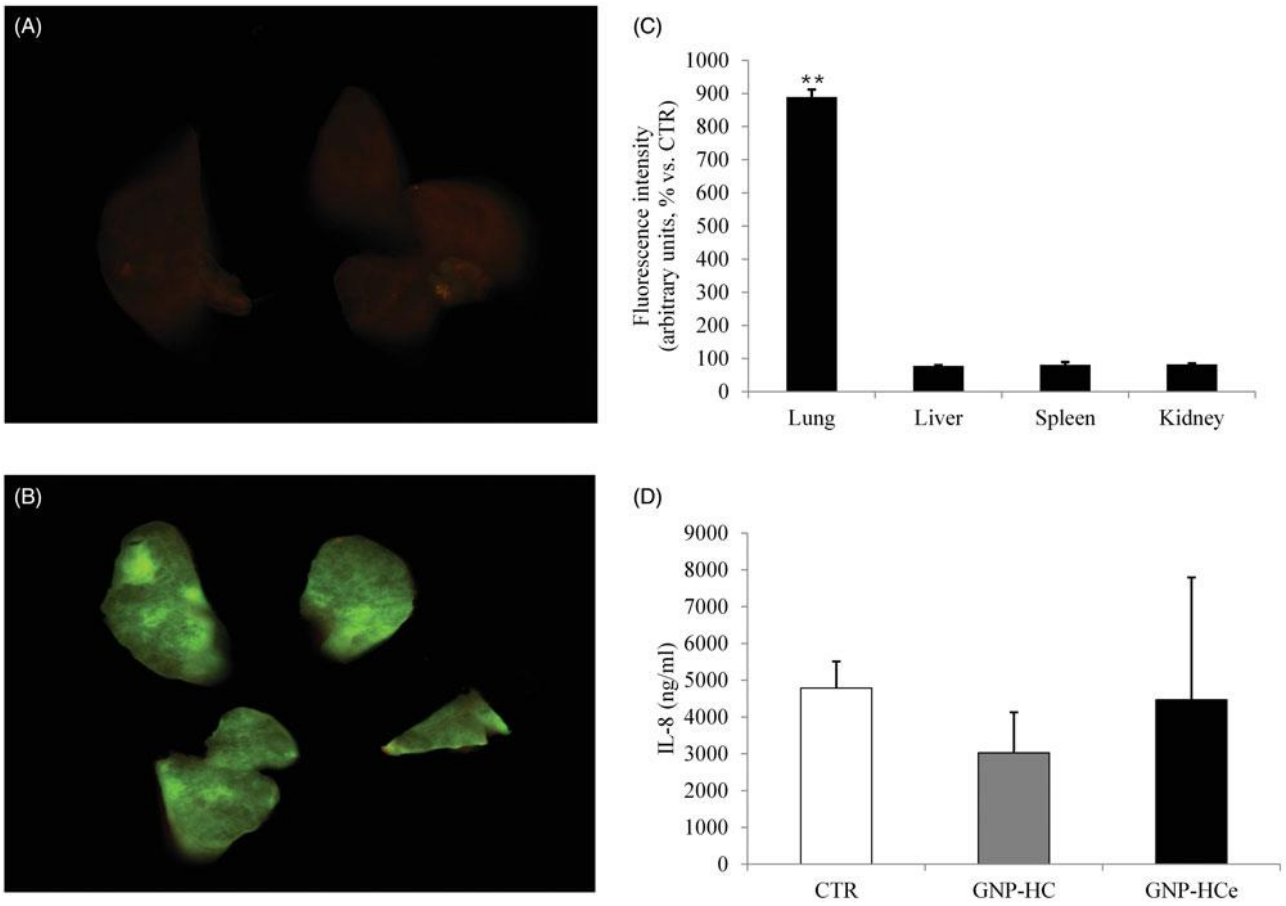

Figure 23 Localization of nanoparticles in lung and peripheral tissues and effect on IL-8 lung production. Representative experiment showing (A) un-treated and (B) treated lungs. A weak auto-fluorescence background (red) is detected in un-treated lungs (A) while lungs inhaled GNP-HC nanoparticles marked with IR-820 dye show a high fluorescence signal (green, B). Fluorescence intensity recorded in different organs (lung, liver, spleen and kidney) of mice inhaled GNP-HC nanoparticles marked with IR-820 dye is evaluated compared to the same organs of un-treated mice (C). IL-8 production assayed in BAL of un-treated and treated (GNP-HC and GNP-HCe) mice (D). Histograms are obtained from the means ± standard error of three experiments. **$p<0.001$ vs. all other tissues. (Modified from Cova et al 2017)

Supported by these latter findings, in collaboration with Columbia University, a method of lung distribution of aerosolized Gold nanoparticles in lungs of rats during mechanical ventilation was assessed, to explore the feasibility of this therapeutic strategy during ex vivo lung reperfusion.
The experiments were performed in vivo on Sprague Dawley rats (7w/250g) endotracheally intubated and sedated with 4-5% isoflurane.
The treatments were performed with saline control, gold nanoparticles and fluorescent gold nanopartles, using Harvard VentElite ventilator connected in series with anesthesia machine and AeroNeb vibrating mesh Lab animal nebulizer (as shown in material section).
At the end of the administration, the system was cut-off for the rat to be awaken, extubated and placed in its cage to recover.
After 24 hours, the rats were re-anesthetized with 4-5% isoflurane and CT scanned using the Quantum FX micro-CT scanner, equipped with internal anesthesia nose-cone, to obtain coronal images of the lungs as well as 3d renderings (figure 24)

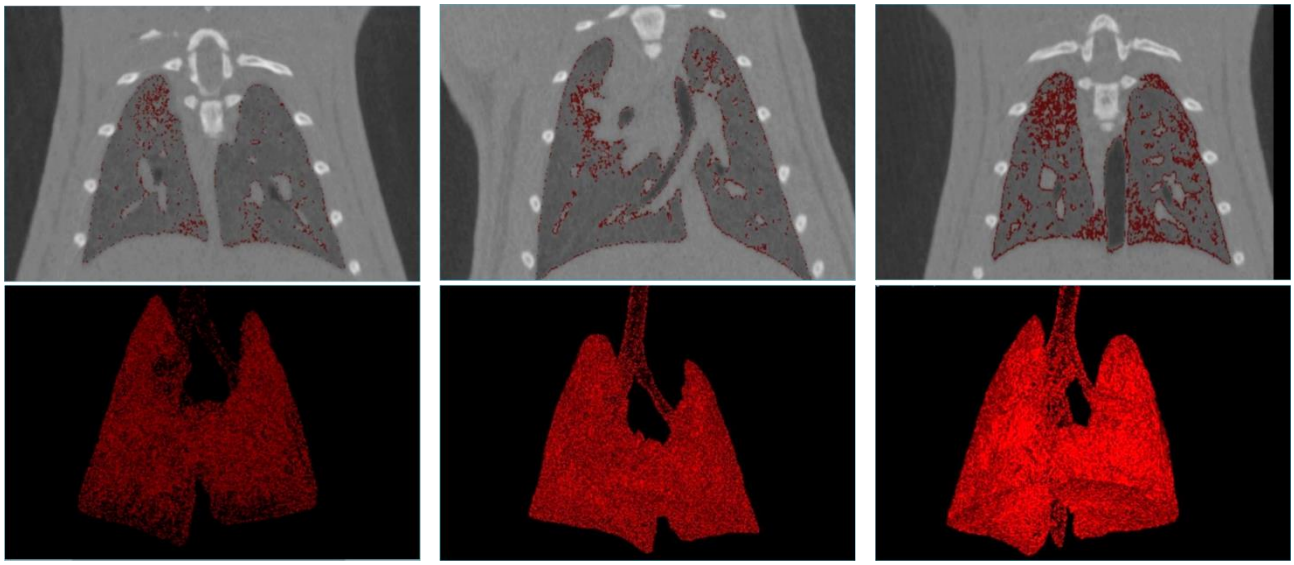

Figure 24. Coronal CT Series Displays Dose Dependent Density of GNPs 3D Rendering of Lungs Displays an Even Distribution. The incremental increase in density of red, which represents the nanoparticles nebulized in the lung and the fact that the delivery was bilateral

Post-scan the rats were euthanized, their organs (lungs, kidneys, spleen and liver) procured and embedded in OCT for storage. The tissue treated with FITC-GNPs was fluorescently scanned to confirm the presence of nanoparticles (Figure 25).
The normal tissue has been stained with DAPI blue for nuclear counter stain and the dark empty space you see in the middle shows an airway. On the right-hand slide, instead, is a similar lung

section cut, treated with our baseline concentration X of fluorescinated nanoparticles. The tissue has again been stained with dapi blue for nuclear counterstain, yet in this case you can easily spot the addition of nanoparticles having a wavelength of 495-519 nm and therefore showing up in green.

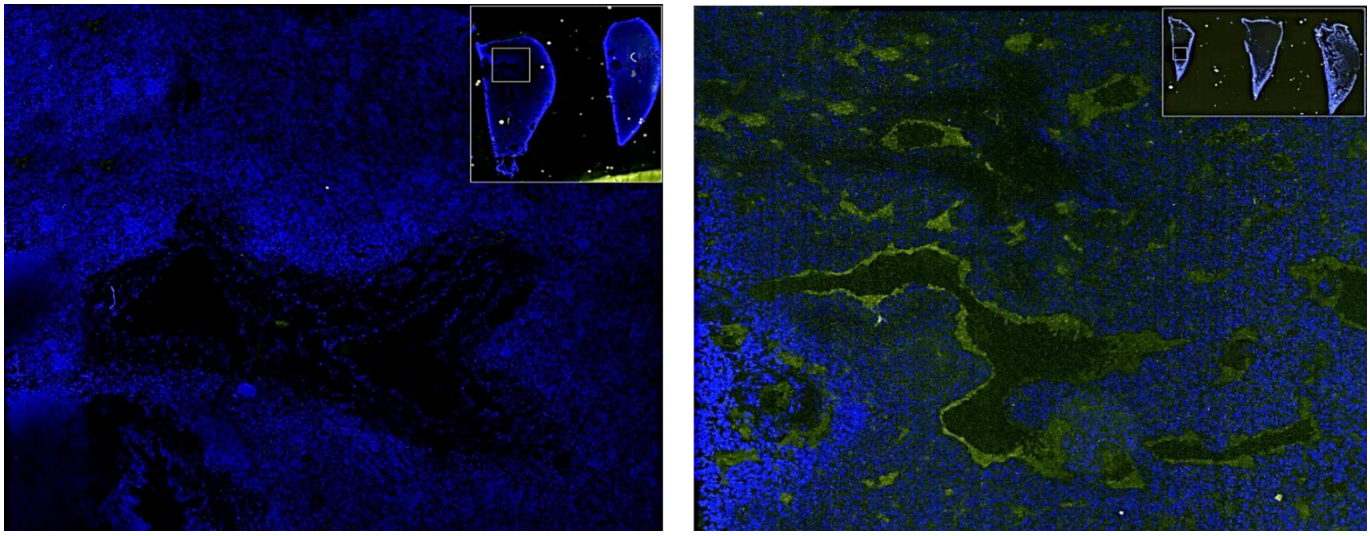

Figure 25. Fluorescent Tissue Scans Display GNP Presence in Treated Lung

DISCUSSION

The identification of all possible risk factors responsible for the development of CLAD is one of the key factors for a better and correct management of patients undergoing lung transplantation.

The recognition of risk factors and their subsequent correction, if possible, certainly have an impact on the quality of life of patients but also on long-term survival after transplantation, preventing the development of chronic rejection. Many risk factor are already known to be relevant in BOS pathogenesis, such as recurrent episodes of acute rejection (Bando et al. 1995, Burton et al. 2008), CMV infection (Patel et al., 2012; Paraskeva et al., 2011; Kotton et al. , 2010; Valentine et al., 2008; Hodson et al., 2008), gastro-esophageal reflux (Goettlieb et al, 2009 and Khalid et al., 2013), time of ischemia of the graft and HLA mismatch (Palmer et al. 2002).

The study by Ruttens et al. which also involved the center in Pavia is the first study investigating the effect of air pollution and traffic exposure in a large multicenter European cohort of lung transplant patients and correlated pollution effects with BOS treatment.

In fact interestingly, the effect of air pollution was not limited to the association with CLAD but showed also an impact on the overall survival. Moreover, this study suggested a protective role of macrolides (Azithromycin) against air pollution. The protective effect of macrolides on air pollution is not entirely unexpected as these drugs are known to reduce innate (neutrophilic) immune activation in many chronic respiratory disorders, such as chronic obstructive pulmonary disease (COPD) (Albert et al, 2011), CF (Saiman et al, 2003), non-CF bronchiectasis (Wong et al. 2012), severe asthma (Brusselle et al, 2013) and CLAD (Vos et al 2011).

Moreover, in lung transplantation patients, PM10 was associated with airway inflammation on transbronchial biopsies and lavage samples, but again only in those patients not taking azithromycin (Verleden et al. 2013).

These findings corroborate the proposed WHO limit for air pollution, as the effect of long-term air pollution exposure was only found among those patients with exposure to PM10 ≥20 µg·m–3. Two-thirds of our lung transplant patients were exposed to PM10 values above the WHO (annual) standard of 20 µg·m–3. The observed associations regarding mortality (13.8% increased risk per 10 µg·m–3 PM10 increase; 6.7% increased risk per 5 µg·m–3 PM10 increase) are somewhat more pronounced than in other similar studies (Fisher et al, 2015).

In this study there was no sub-phenotyping of CLAD (BOS or RAS) as there were no uniform diagnostic criteria and, even so, using these categories on a large retrospective cohort was very difficult. The findings of this study suggest that a significant association exists between air pollution and mortality/CLAD in lung transplant patients and envisage further investigation on the possible

protective effect of macrolide therapy against the detrimental effects of air pollution.

Another possible risk factor that has been studied is the hystopathological features of donor lungs, in fact the quality of donor organs may have an impact on early and long-term outcome after lung transplantation. Donors are usually evaluated according to clinical, functional, radiological and endoscopic features (Orens et al, 2003).

The histopathological abnormalities are never considered in the evaluation due to logistic reasons and the need to start lung ischemia interval. In our study, we retrospectively analyzed pathology reports of transplanted lungs undergone downsizing by non-anatomical lung resections at the time of transplant, performed at Lung Transplant Unit at NYP/Columbia University of New York. Our aim was to evaluate the role of graft anatomical abnormalities at the time of transplant on long and short tern outcomes. Results showed that alveolar (emphysema), bronchial (bronchiolitis) or vascular (thrombi) abnormalities are present in most of cases (60% on transplanted lung). However, lung pathology does not seem to affect overall survival, presence of CLAD (either BOS or RAS), infections rate, acute rejection rate or lung function after lung transplantation, in terms of best FEV1 after transplantation. We can therefore conclude that histo-pathological evaluation of lung tissue (unlike other transplant settings as kidney or liver) does not add clinically useful information for the outcome of lung transplantation.

Moving forward on the risk factors of BOS onset analysis, from the immunological point of view, it is well known that multiple episodes of acute rejection are the major risk factor for BOS development.

The exact pathogenesis of BOS is not well understood, but is thought to be the result of a chronic immune and inflammatory reaction that involves an exaggerated reparative fibrotic response. Some debate is still present on the “net ” role of allo-specific immunity (either reaction or tolerance) and aspecific inflammatory factors in the pathogenesis of BOS..

Although recent evidence ascribes an important pathogenic role to non-specific inflammatory mechanisms, the relevance of specific immunity has been clearly established (Vanaudenaerde BM et al 2008, Evers et al 2015). Effector T cells have long been recognized as critical mediators of rejection and regulatory T cells ($CD4^{+}CD25^{high}CD127$ T-reg) as mediators of tolerance (Issa et al 2013).

These data have been partially confirmed in kidney and liver Tx recipients, showing a positive correlation between graft survival and the number of circulating CD4+CD25^{+} T-reg cells, as well as a correlation between their peripheral fluctuation and the occurrence of acute and chronic rejection (Zhang et al 2015, Cretney et al 2013).

As for lung Tx, evidence is limited and somewhat contradictory (Neujarh et al 2015). In previous cross- sectional studies, it has been showed that lung Tx recipients with BOS had significantly lower peripheral CD4+CD25high T-reg cells than clinically stable lung recipients, and demonstrated their functional regulatory profile, in vitro (Meloni et al 2006, Meloni et al 2004). On the other hand, subsequent studies failed to demonstrate a correlation between Treg cell counts and long term lung Tx outcome (Nakagiri et al 2012, Krustrup et al 2014), except a recent study which stated that higher frequencies of specific Treg subpopulations early after lung transplantation are protective with respect to the incidence of CLAD at 2 years (Salman et al 2017).

The presented study (Piloni et al 2017) is the first longitudinal study reporting the long-term kinetics of peripheral CD4^{+}CD25^{high}CD127^{-} T-reg cells in lung recipients, and suggests that peripheral T-reg cell counts can predict CLAD onset/progression. It has been established that demonstrated that CD4^{+}CD25^{high}CD127^{-} subset is significantly enriched with FOXP3+ cells, thus inferring that it is endowed with regulatory functions. Moreover, thanks to the large number of determinations included in this long term study it has been demonstrated that:

1) peripheral counts of CD4^{+}CD25^{high}CD127^{-} T-reg cells significantly decrease in CLAD patients;

2) the degree of their decrease is associated with the severity of CLAD and, most noteworthy;

3) CLAD onset or BOS GRADE is significantly associated to mean T-reg cell counts in the previous trimester.

Finally, the association of CD4^{+}CD25^{high}CD127^{-} T-reg cell counts with a number of clinical variables was tested, and, interestingly, only azathioprine and ECP were found to be significantly associated to them at the univariate analysis, while, in the multivariate model apart CLAD no other variables were associated to T-reg cell count.

Interestingly, unlike previous evidence in literature (Wang et al 2013, Levitsky et al 2013) we could not detect any significant variation of T-reg cell counts with respect to other specific immunosuppressive drugs such as cyclosporine A, tacrolimus or everolimus, nor the effect of lympho-depleting strategies, since induction treatment is not routinely performed at our center.

This work is the first observation on a large cohort of patients to confirm an association between

Treg cell counts and CLAD onset/progression, including both BOS and RAS phenotype.

Besides regulatory T cells, some recent reports highlighted the role of B cell regulatory subsets as possible mediators of graft acceptance (Barthelot et al 2013, Dijke et al 2016). Within B -regulatory populations (B-reg): the subset of immature B-cell, phenotypically characterized as CD19+CD24highCD38high has been described at peripheral level in healthy individuals (Blair et al 2010, Mauri et al 2015). This subset is characterized by the release of high amount of IL10 (much higher than other Breg cell subsets) and the ability to suppress Th1 and Th17 differentiation (Flores-Borja et al 2013) and convert CD4+ T cells into T-regs and Tr1 cells (Mauri et al 2016)

Evidences on the role of This Breg cell subset in transplant acceptance are still scarce.

As for lung transplantation, the role of B regulatory cells has not yet been investigated in vivo, the only evidence being a report on a murine tracheal transplantation model showing that rapamycin was able to prevent long term tracheal obliteration through a massive tracheal infiltration of regulatory B cells (Zhao et al 2013).

In the study performed by Piloni et al (submitted in Transpl Immunology, 2018), it has been clearly demonstrated on 5 blood samples of representative samples from lung transplant recipients, that CD19+CD24highCD38high B cell subset is highly enriched with IL10 producing cells thus suggesting that most (> 90%) of these cells are endowed with regulatory functions. By the paracrine IL10 effect B-regs have been reported to inhibit Th1 responses via IL-10 and Th17 differentiation, in addition CD19+CD24highCD38high B-regs could also convert “in vitro” CD4+ T effectors into T-regs and Tr1 cells (Mauri et al 2015, Flores Borja et al 2013).

There is scanty experimental evidence that that CD19+CD24highCD38high B-reg cells might be implicated in transplanted graft acceptance, mainly gained in the setting of kidney transplantation. A recent report on a small cohort of kidney recipient, showed that immunosuppressive regimen based on a combination of mTOR- and calcineurin inhibitors reduces CD19+CD24highCD38high B-reg cell counts (Latorre et al 2016).

Thus the real role of CD19+CD24highCD38high B-reg cell subset “in vivo” is still undefined. This regulatory aspect remains poorly characterized in human clinical transplantation.

Thus for the first time, in this work counts of CD19+CD24highCD38high B-reg cells in the peripheral blood of lung transplant recipients was analyzed and correlated with a number of clinical and therapeutic variables.

On the basis of present study, we can infer a role for CD19+CD24highCD38high B-reg cell subset in the long term acceptance of lung graft since we specifically found a slight downregulation in

presence of CLAD irrespective of its phenotype.

Furthermore, an interesting observation was that CD19+CD24highCD38high B-reg cell counts are significantly affected, during specific bacterial and fungal infections (*S. aureus* and *A. fumigatus*). Even if the small number of samples obtained during *S. aureus* and *A. fumigatus* infectious episodes might have limited the strength of the statistical analysis. Recent experimental data highlighted that pro-inflammatory stimula induced by a specific gut microbiota were able to expand B-reg cell number and function and to restrain inflammatory reaction associated to antigen-induced arthritis in mice (Rosser et al, 2014). Unfortunately, we could not study an association with lung or gut microbiota but this issue needs further evaluation in future specifically designed surveys.

Unlike previous evidence in literature (Lund et al 2010), we could not detect any significant variation of B-reg cell counts with respect to other specific immunosuppressive drugs such as m-TOR inhibitors, however a limited number of patients submitted to other immunosuppressive regimen than our typical (tacrolimus, micophenolate mofetil and steroids) have been enrolled. Of note also in this study, we could not assess the possible role of lympho-depleting strategies, since induction treatment is not performed at our center.

Finally, since an interaction between B and T-reg cells via IL-10 has been reported elsewhere (Latorre et al 2013), we decided to verify this hypothesis in our cohort. As shown in the results section, we could not confirm this observation in humans and found no association between the $CD19^{+}CD24^{high}CD38^{high}$ B-reg and the $CD4^{+}CD25^{high}CD127^{-}$ T-reg cell subset. We cannot exclude however that other T reg subset such as Tr1 (that were not addressed in our study) might have been regulated by $CD19^{+}CD24^{high}CD38^{high}$ B-reg cell in our patients.

This report on peripheral CD19+CD24highCD38high B-reg cell kinetics in lung recipients highlights, for the first time, that this cell subset might participate in long-term lung graft acceptance mechanisms and that the counts of these B reg cells are significantly influenced not only by type of immunosuppressive regimen but also by specific infective complications.

All together our studies stress that the role of allospecific immunity and tolerance in the pathogenesis of BOS is relevant, thus point out the need for more appropriate, tailored, safe and less toxic therapeutic intervention in the prevention and treatment of BOS.

A cornerstone of preventing allograft rejection in solid organ transplantation is the immunosuppressive treatment. It can be schematically declined in 2 phases. The initial phase, related to the induction, serves to rapidly depress adaptive immunity and the second phase corresponds to the maintenance therapy, which inhibits global lymphocytic (T- and B-cells) proliferation by different and complementary mechanisms.

The use of induction therapy, whatever the modalities, has shown a benefit on the prevention of early acute cell rejection (Hachem et al 2008, Scheffert et al 2014). Nevertheless, the induction immunosuppressive therapy remains controversial and is used by only about half of the lung transplant teams through the world (Yusen et al 2015), because is associated to a significant risk infections and to a high risk of long-term malignancies. The 2 mains validated therapeutic options targeting T-lymphocytes includes anti-thymocyte globulins (ATG) and basiliximab, a chimeric monoclonal antibody targeted against the alpha chain of the T-cell IL2 receptor (CD25).

The impact on the immune system and the kinetics of peripheral lymphocyte population reconstitution has never been compared according to the types of induction prior to the the study from Coiffard, Piloni et al 2018. Were included patients who underwent a first LTx in 3 different lung transplant centres in Europe with different induction strategy (no induction – Pavia, anti-thymocyte globulins - Marseille and basiliximab - Strasbourg)

This study provides new information on the kinetics of circulating lymphocytes and lymphocyte reconstitution within 2 years following a lung transplantation. In this study only recipients with the same up-to-date maintenance treatment consisting of tacrolimus, mycophenolate mofetil and corticosteroids were included. Analysis of lymphocyte kinetics in our whole transplant cohort shows an early and maximal effect of the induction therapy at day 7. We then note a phase of prolonged lymphocytic reconstitution, without return to the initial pre-Tx values probably related to the maintenance treatment.

As expected, the induction modalities influence the lymphocyte course. Induction with ATG or basiliximab results to a higher lymphocytes suppression in the early post-operative phase, but this effect seems also persist in the time with significant differences during the reconstitution period.

This study also showed that pre-Tx weight, which is not correlated with lymphocyte level before LTx, strongly impacts the immunosuppression effect in the reconstitution phase; recipients with small weight in pre-Tx had lower lymphocytes all long the study period. Undernutrition is associated with suppression of the antigen-specific arms of the immune system, decreased T-lymphocyte proliferation, and atrophy of the lymph tissues, but many of these data are from children and the

relevance to immune recovery in transplantation is unknown (Keusch et al 1993, Najera et al 2007). Even more interesting, this results shown a strong relation between level of lymphocytes in pre-Tx and post-Tx. Indeed, despite no difference at day 7, recipients with lower lymphocytes in pre-Tx persist at a lower level during all the reconstitution phase, which predicts an immune profile after transplantation. Finally, in the reconstitution phase, lymphocytes demonstrated a prognostic value by being link with mortality with a best predictive performance at day 90. This result suggests that as induction therapy is necessary to prevent acute rejection, immune reconstitution is as important and fundamental to the long-term allograft outcome, and supports the idea of personalizing immunosuppression.

As for the maintenance treatment, the challenge is to achieve in each single patient, a good balance in the immune response, minimizing both the risk of infection and rejection, since under-immunosuppression leads to an increased risk of rejection while over-immunosuppression leads to an increased risk of infection.

Therefore, one of the most important strategies to ensure the long-term viability of the allograft is the careful monitoring of immunosuppression, which can reduce the incidence rate of infections and rejection (Sangeeta et al 2015).

Until a few years ago, only the assessment of drug trough levels in the venous circulation, clinical manifestations or drug toxicity were used to determine the adequacy of drug dosage, despite the fact that the blood concentration of IS drugs does not predict the rate of biological effectiveness (Jon et al 2010). In addition, these methods are not tailored to each individual and do not necessarily assess the appropriate levels of IS for a given individual. Specifically, pharmacokinetics often differ among individuals due to clinical factors such as underlying diagnosis, age, gender and race.

The major challenge in lung transplantation is balancing immunosuppression in order to avoid drug toxicities and complications of over- and under-immunosuppression. During the past years, several clinical trials of the use of IMK have shown that it is able to detect and measure the rate of "over-IS" of CD4+ cells in solid organ transplant patients, such as the kidney, heart, small bowel and pancreas. All these studies demonstrated the promise of IMK in solid-organ transplant recipients, with the potential for modulating immunosuppression based on the individual patient's risk profiles for infection.

ImmuKnow assay (IMK), approved by the Food and Drug Administration in 2002, is the only patented test for the assessment of cell-mediated immune response in immunosuppressed patients. The assay evaluates the ability of helper T-cells to respond to the mitogenic capacity of

phytohemagglutinin (PHA) *in vitro*, quantifying adenosine triphosphatase (ATP) production by the CD4+ population (Kowalski et al 2003).

In our Institution the Immuknow assay has been used since 2010 and on the basis of literature data, it was decided not to use the test at regular intervals but only at one specific time point (6 months post- transplant) plus on an "as-needed basis": i.e., in the case of suspected IS /infectious risk, and in case of the need to down titrate down the immunosuppression due to toxicity such as kidney failure or bone marrow toxicity with cytopenia.

We registered a considerable number of infection episodes, thus finding that IMK levels below the cut-off of 226 ng/ml were significantly associated to the presence of infection: lower levels of IMK assay were, in fact, more frequently detected in infected patients compared to non-infected patients.

However, since our multivariate analysis conducted on single point samples demonstrated that IMK values below cut off of 226 ng/ml are significantly associated to infection, we can speculate that, also a single time IMK analysis might be clinically useful to detect a condition of over IS, possibly associated to infection. This helps to resolve contrasting findings in the literature, thus confirming the clinical utility of this assay in case of IS, to predict risk of infections.

Surprisingly, in our population we found a strong association between the presence of RAS and low IMK level. A possible explanation for the observation that low IMK levels might be related to higher IS rates (lower IMK values) in RAS than in BOS, might be found in the use of more aggressive IS therapeutic strategies in RAS than in BOS, with the aim of slowing down the progression of this rapidly evolving CLAD phenotype, which is thought to be associated to a higher degree of graft inflammation (Verleden et al 2014).

This retrospective study confirms the utility of IMK assay in lung transplant management, especially with the aim to individually tailor the IS rate to single patients and thus reduce the risk of over-IS, which is also enforced by the observation that in a high percentage of our cases results of the IMK test drove clinical decision on the adjustment of IS.

Another relevant issue concerning immunosuppressive treatment, as already stated, is that current immunosuppressive regimen, even if carefully adjusted and monitored are not able to prevent the

onset of BOS, whose prevalence at 5 years post-Tx is still very high (>50% according to ISHLT registry). Moreover, no treatment is able to revert obliterative lesions, once occurred, and current therapeutic approaches (including ECP) are at best able to stabilize graft function in almost 60% of patients for some months/years.

All current drugs are scarcely effective, also because systematically administered and associated to unpredictable biodistribution, insufficient drug accumulation in the thorax, limited efficacy and systemic toxicity; thus we exploited the possibility of a new drug delivery system.

In the previous work, Cova et al. proved the in vitro efficacy of gold nanoparticles loaded with an immune-suppressive/anti-proliferative drug (everolimus) specifically targeted against pulmonary mesenchimal cells, the main effectors driving the fibroproliferative obliteration of bronchioles, to inhibit MC without affecting the viability of bronchoalveolar epithelial cells.

Given the multistep pathogenic process that lead to BOS, inflammatory cells, alveolar macrophages, neutrophils and lymphocytes are key effectors in fibrogenic process. Thus, it was mandatory to exclude a proinflammatory activity of gold nanoparticles and assess whether an anti-inflammatory activity was present.

Therefore, experiments in vitro either using unstimulated isolated cells were performed (Cova et al, 2017) and after a proper stimulation, the latter condition mimicking an already activated/inflamed environment.

As for macrophages, no stimulatory effect or increased oxidative stress was observed by treatment with GNPs decorated with the antibody either under basal or activated conditions, as previously described for gold nanocarriers (undecorated GNPs) (Shukla et al., 2005). In contrast, a reduced macrophage viability was recorded with GNPs loaded with everolimus (either coated or not with anti CD44), demonstrating that the toxic effect was actually due to the drug release into the phagocytes. Similar results were obtained with neutrophils.

In analogy to previous findings (Uchiyama et al., 2014) we did not detect a significant pro or anti-inflammatory activity of “nude” GNPs. Although, a considerably higher inhibitory activity of GNP-HCe was detectable toward lymphocytes, in particular, IFN-g and IL-17 secretion by stimulated T cells, was markedly downregulated, and thanks to the pivotal role of these cytokines in BOS pathogenesis this result is particularly relevant.

After the achievement of these encouraging in vitro data showing the lack of a proinflammatory activity of our engineered GNPs, we moved to assess the *in vivo* (on healthy mice) localization and toxicity. It has to be noted that our delivery schedule included 30-min inhalation a day for up to 2

weeks. The results confirmed that inhaled nanoparticles did not rise the inflammatory response at the pulmonary level, did not even induce significant toxicity and seemingly localized mainly into alveolar space due to capture by macrophages. In another work, a single intratracheal instillation of 50 and 250nm GNPs generated only a mild inflammatory response that was mostly localized in alveolar macrophages (Gosens et al., 2010). The absence of inflammatory response observed by us in lung tissues could be ascribed to aerosolized route of administration which is usually associated with a higher dispersion rate, compared to a previous work (Gosens et al., 2010), where a single intratracheal instillation of 50 and 250nm GNPs generated only a mild inflammatory response that was mostly localized in alveolar macrophages.

Another key point of this experiment was the lack of fluorescence and gold signal in peripheral tissues, is in agreement with previous reports in which GNPs with similar size locally administered by inhalation or intratracheal instillation were mostly retained in the lungs (Balasubramanian et al., 2013; Gosens et al., 2010; Han et al.,2015; Kreyling et al., 2014).

The use of nanoparticles in the attempt to solve urgent medical need represents a huge challenge and this work discloses the possibility to extend the use of this innovative tool to prevent/revert bronchiolar fibro-obliteration and the onset of chronic rejection after lung transplantation, namely BOS. In vivo experiments comply with in vitro observations and, demonstrating the feasibility of the local inhalatory administration route, allow to rationalize the dose schedule and the number of animals to be used in future experiments aimed to assess therapeutic activity in BOS models.

As a further step ahead in this project, during the 4-months fellowship at Columbia University, in order to develop a drug delivery approach assuring an even and safe bilateral distribution of GNPs in lungs, ascertained a non-invasive method of detection for GNPs in lungs and monitor time-dependent and dose-dependent particle deposition. An in vivo experiment on anesthetized rats was performed and by this experimental inhalation method, we intubated healthy rats and administered our GNPs diluted in saline with a nebulizer directly into the trachea.

This method was successful to deliver nanoparticles throughout the lungs, with a CT-based detection system was performed a non-invasive quantitative analysis of nanoparticle distribution in the lungs displaying an even and bilateral distribution. Furthermore, the nanoparticle delivery and deposition within the lungs showed an dose-dependent effect.

These findings are encouraging and allow us to envisage the need of future experiments on BOS animal models in order to assess the efficacy of GNP-HCe in preventing/ reverting chronic rejection

considering that nanoparticles uptake and behavior might be different in diseased lungs, as previously described (Geiser et al., 2013).

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