Diplomarbeit

Tissue-specific, Auto-reactive CD4CD28null cells in Explanted COPD Lungs

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1.1 Abstract

Chronic Obstructive Pulmonary Disease (COPD) is a form of lung disease, and one of the leading health issues worldwide; with predictions that it will become the third leading cause of death by 2030. The major risk factor for the development of this disorder is smoking, through direct tobacco use or second-hand smoke. Patients with COPD suffer from a chronic inflammation of the lung, leading to progressive limitation of the airflow through a persistent blockage of airways. Although this life-threatening disease leads to the death of more than 3 million people every year, it is still under-diagnosed; and the pathogenic pathways are still vaguely described.

In 2003 it was proposed that COPD might have autoimmune components. This statement is supported by a couple of facts. Firstly, only a small percentage of smokers reach the later stages of COPD. Secondly, the disease progresses despite smoking cessation, and lastly, that Smokers have increased levels of antigen-presenting cells. CD4+CD28null cells define a specific pro-inflammatory T cell subset. Several studies were able to detect these cells, not only in patients with chronic inflammation and acute coronary syndrome, but also in autoimmune diseases such as Rheumatoid arthritis, Wegener's granulomatosis, Ankylosing spondylitis, Multiple sclerosis, and Inflammatory bowel disease. Research on CD4+CD28null cells showed that because of chronic stimulus the cells lose the co-stimulatory molecule CD28, contain Perforin and Granzyme B, and are able to lyse target cells upon activation of killer immunoglobulin-like receptors (KIRs), a multigenic NK-receptor family. These cells revealed to be highly resistant against pro-apoptotic signals, thus making it likely that they play an important role in autoimmune diseases.

This study was intended to investigate the role of CD4+CD28null cells in the pathogenesis of COPD. We evaluated lungs from end-stage COPD patients and compared the levels of tissue infiltrating CD4+CD28null cells with systemic levels. We could show that CD4+CD28null cells were present in high amounts in lung tissue obtained from explanted COPD GOLD IV lungs, suggesting a direct involvement of those cells in the pathophysiology of COPD. Furthermore, purified lung-resident CD4+ cells showed a stable proliferative response to lung specific elastin and collagen. These results further corroborate the role of autoreactive CD4+ cells in the maintenance of the inflammatory destruction happening in COPD. Modulating CD4+ cell function might be a new promising tool for future therapeutic approaches.

1.2 Zusammenfassung

Chronic Obstructive Pulmonary Disease (COPD) ist eine chronische Lungenerkrannkung und eine der führenden Gesundheitsprobleme weltweit. Statistische Vorhersagen gehen davon aus, dass diese Erkrankung die dritt häufigste Todesursache sein wird bis 2030. Der größte Risikofaktor für die Entstehung dieser Erkrankung ist Rauchen, sowohl direkter als auch indirekter Tabakkonsum. Patienten mit COPD leiden unter einer chronischen Entzündung der Lunge, welche zu einer fortschreitenden Einschränkung der Atmung führt, bedingt durch anhaltende Blockierung der Atemwege. Obwohl diese lebensbedrohliche Erkrankung zu mehr als 3 Millionen Toten jährlich führt, bleibt die Pathophysologie ungenügend verstanden und unterdiagnostiziert.

Eine Studie aus dem Jahr 2003 formulierte die Idee das COPD möglicherweise auch Eigenschaften einer Autoimmunerkrankung aufweist. Diese Behauptung wird durch einige Fakten unterstützt:1) nur ein geringer Prozentsatz der Raucher erreichen das Endstadium der Erkrankung 2) das COPD bei manchen Patienten trotz Rauchabstinenz fortschreitet 3) Raucher erhöhte Werte antigenpräsentierende Zellen aufweisen. CD4+CD28null Zellen bezeichnen eine spezielle Untergruppe von pro-inflammatroischen T-Zellen. Diverse Studien konnten diese Zellpopulation in Patienten mit chronischen Entzündungen, akutem Koronarsyndrom aber auch in Autoimmunerkrankungen, wie zum Beispiel Rheumatoide Arthritis, Granulomatose mit Polyangiitis, Spondylitis ankylosans, Multiple Sklerose und in Chronisch-entzündliche Darmerkrankungen, nachweisen. Studien konnten zeigen, dass CD4+CD28null Zellen eine hohe Resistenz gegenüber Signalwege welche Apoptose induzieren, aufweisen und, dass diese Zellpopulation daher eine wichtige Rolle in Pathomechanismen von Autoimmunerkrankungen spielt.

Forschungsarbeiten zum Thema CD4+CD28null Zellen zeigten, weiters dass durch chronische Stimulation das membranständige CD28 Molekül herunterreguliert wird und Perforin, sowie Granzym B in den Zellen exprimiert werden. Des Weiteren besitzen diese Zellen einen Killer Cell Immunoglobulin-like Receptor (KIR). KIR ist ein Rezeptor, der hauptsächlich in Plasmamembranen von natürlichen Killerzellen (NK-Zellen) vorkommt. Wenn es zu einer Aktivierung dieses Rezeptors kommt, sind sie in der Lage sind Zielzellen zu lysieren.

Diese Studie hatte das Ziel, die Rolle der CD4+CD28null Zellen in der Pathogenese von COPD zu untersuchen. Wir untersuchten explantierte Lungen im Endstadium einer COPD und verglichen die Menge an CD4+CD28null Zellen im Lungengewebe mit der systemisch nachweisbaren Menge.

Wir konnten zeigen, dass CD4+CD28null in hohen Mengen im explantierten Lungengewebe von Patienten mit COPD Stadium GOLD IV nach LTx zu finden waren. Dies macht es wahrscheinlich, dass es einen direkten Einfluss dieser Zellen in der Pathophysiologie der Erkrankung COPD gibt. Zudem zeigten aufgereinigte CD4+ Zellen dieser COPD Patienten eine nachweisbare proliferative Reaktion bei Inkubation mit lungenspezifischem Kollagen und Elastin. Diese Ergebnisse bekräftigen die Behauptung, dass Autoreaktive CD4+ Zellen die durch die Entzündung hervorgerufene Zerstörung des Lungengewebes aufrechterhalten. In der Zukunft könnten Therapien die die Funktion von CD4+ Zellen verändern ein vielversprechender neuer Ansatz werden.

2. Background

2.1 Epidemiology of COPD

Chronic obstructive pulmonary disease (COPD) is a worldwide burden effecting developed and developing countries alike. This disease is the most common lung disorder, with estimations of 64 million people affected. In 2002 the WHO ranked this disease as the fifth leading cause of death, and predictions show that by 2030 it will reach the top three [2]. As shown in many studies, the major risk factors for the development of this disorder are smoking, through tobacco use or second-hand smoke, and indoor air pollution. These facts highlight that COPD is a global burden, and that it is preventable.

Patients suffering from this disease experience worsening limitations of their expiratory airflow through progressive blockage of the airways. Although it is well established that COPD is caused by a chronic inflammation of the lung tissue, the exact pathogenesis pathway is still uncertain.

2.2 COPD as a systemic disease

The majority of patients that suffer from COPD do not end up dying of the disease. Cardiovascular disease, type 2 diabetes mellitus, and lung cancer are the disorders with the highest mortality rate associated with COPD [3, 4]. Several studies were able to provide evidence that COPD causes an increase of pro-inflammatory cytokines. TNF-α, IL-6, Il-8, and C-reactive protein (CRP) are proteins which are already described as being detectable in elevated concentrations in this circulation of patients with this disease. These findings were especially significant in patients during exacerbation, but were also measurable in patients that seemed clinically stable [5].

2.2.1 Systemic effects – Weight loss

The inflammatory response that is happening in COPD is also associated with increased numbers of activated neutrophils, monocytes, and lymphocytes in peripheral blood [6, 7].

A study that took a closer look at the monocytes showed that those cells, that were harvested from COPD patients, generate higher levels of TNF-α when stimulated than those of controls. Interestingly, there was a correlation between the expression of TNF-α and the body weight of the patients. The higher the protein secretion, the lower the body weight of the COPD patient [8]. This is not the first study that described an effect of COPD on body weight. Unexplainable weight loss is present in about 50% of patients with late stage COPD, and in up to 25% in patients with moderate airway obstruction [9]. Studies found differences in caloric intake, metabolism, and changes in body composition. It is important to know that changes in body composition can appear even without major differences in weight. Hence simple weighing is not sufficient to detect these changes, technics like bioelectrical impedance measurement and bone densitometry scans are necessary. With these technics significant differences in lean body mass, fat mass, and bone mineral content were found, when comparing healthy controls with patients suffering from COPD or chronic bronchitis [10]. Studies that ascertained the nutritional status of COPD patients found that they generally appear in cachectic. Their caloric intake is slightly increased, metabolic rate is elevated, and they hardly benefit from nutritional support [11, 12]. The reason for the increased metabolic rate is not entirely certain. One possible explanation is that because of the heightened stress and breathing effort, the respiratory muscles are more demanded, thus needing more energy [13]. Other mechanisms that could induce these changes in metabolic rate are: 1) drugs like β_2-agonists, which are administered for treatment of COPD, are known to have an effect on metabolism 2) a general hypoxia 3) systemic inflammation. The latter two induce cell stress increasing energy expenditure throughout the body [14].

Several studies that analysed the body weight of COPD patient during their treatment were able to show that it functions as a reliable prognostic marker [15]. This factor is independent of airway obstruction and helps assess the general health of patients. One retrospective study that investigated the outcome of COPD patients showed that patients who regained their lost weight had a significant better rate of survival. IT is important to note is that this was also true for patients that had no improvement in their lung function [16].

Most COPD patients experience a reduction in exercise capacity that worsens with the severity of the disease. It seems obvious that this is because COPD is a pulmonary disease that leads to the obstruction of the airways and dyspnoea.

However, some researchers argue that this is not the sole reason. Many patients who are suffering COPD are complaining about leg fatigue as a factor for their intolerance towards exercise [17, 18]. These findings suggest that COPD is inducing skeletal muscle dysfunction, and there are several mechanisms that could play a role. Chronic hypoxia in the muscle tissue leads to a reduction of protein synthesis, which in further consequence lowers the ratio of myosin in the composition of skeletal muscles [19]. Further is a reduction in muscle tissue, also observable in healthy test subject when exposed to hypobaric hypoxia [20]. These observations show that oxygen therapy is crucial in treatment of patients with chronic respiratory failure.

As mentioned above the systemic inflammation in COPD induces increased levels of TNF-α in patients. TNF-α is able to affect skeletal muscle cells directly through several mechanisms: degradation of myosin, causing sepsis and inducing apoptosis [21-23]. Skeletal muscle biopsies obtained from COPD patients showed increased levels of apoptosis which can be linked to the high levels of pro-inflammatory cytokines in the circulation [24, 25]. Another possible pathomechanism for skeletal muscle dysfunction could be induced through oxidative stress. Not only does it lead to a breakdown of proteins in cells, it is also a crucial factor in the aging process of the body, and maybe be responsible for premature muscle loss [26, 27]. Tobacco smoke is known to be the most important risk factor for the development of COPD but its systemic effects are often overlooked. Studies have shown that tobacco smoke increases the risk of coronary artery disease and arterial endothelial dysfunction [28, 29]. Therefore, a negative effect on blood supply of muscles seems plausible, which in further consequence can induce a degradation of skeletal muscles. Since COPD has also shown to have an influence on the hormone homeostasis, by lowering testosterone and growth hormone concentrations. This could in the longer term also induce a loss of skeletal muscles [30, 31].

Several of these mechanisms not only affect the muscles but also bones. Patients suffering from COPD have an increased prevalence to develop osteoporosis [32]. The most obvious causes for these osteoskeletal changes among COPD patients are malnutrition, decreased exercise tolerance, inflammation and treatment with steroids [33]. Many pathomechanisms of skeletal muscle dysfunction in COPD are not yet fully examined. A combination of various mechanisms is most likely to induce this loss of muscle tissue, which is crucial for the patient’s outcome and quality of life.

2.2.2 Systemic effects – Oxidative stress

Further evidence for a systemic inflammation is the detection of oxidative stress. There are two ways how oxidative stress can be caused in COPD: 1) by intake of oxidants, for example directly through smoking tobacco 2) released by cells of the immune system activated through an inflammatory stimulus, which is present in COPD as mentioned above. Oxidative stress is defined by an accumulation of Reactive oxygen species (ROS).

ROS can damage cells by damaging DNA, oxidizing fatty acids, amino acids and specific enzymes, which lead to their inactivation. Too high amounts of oxidative stress will trigger programmed cell death. Since ROS are highly reactive and instable, their biological half-life is extremely short (between 10 to 10^{-9} seconds). This makes detection in vivo hardly feasible. As an alternative, it is possible to trace the biological reactions and consequences. Two technics to do so are based on this concept are measurement of the Trolox equivalent antioxidant capacity, and the levels of remnants of lipid peroxidation. A study that used these two methods was able to find a significant increase in smokers and COPD patients [34]. Similar results were found by measuring a specific isoprostane that is produced by perioxdation of arachidonic acid through ROS and excreted in urine [35]. Both studies found especially high evidences for oxidative stress in COPD patients in times of exacerbation.

2.2.3 Systemic effects – Nervous system

Observations of the nervous system found changes in the metabolism of the central nervous system [36]. Similar to the effect on muscle cells, these alterations could be caused by chronic hypoxia. Further possible evidence for an effect on the brain is that depression is more common among people who suffer from COPD than healthy persons [37]. It is possible that this is a psychological manifestation of a chronic disease. However TNF-α and other cytokines, that are elevated in the systemic inflammation caused by COPD have shown to play a role in the development of depression [38]. All these findings indicate that COPD is a disorder that affects the whole body, and show that it is crucial that clinical assessment of patients must be thorough. In addition should the systemic symptoms also be included when considering the therapeutic options.

2.3 Exacerbation of COPD

By definition of the Global Initiative for COPD an exacerbation is an acute worsening of the diseases accompanied by increased dyspnoea, elevated production of sputum and deterioration of lung function [39]. The cause for this sudden aggravation of the disease is often an interaction between the patient's immune system, bacteria and viruses. Studies suggest that in most of the times this effect is induced by contact with a new strain of microbe which resulted in an inflammatory response of the host [40]. The most common trigger among the bacteria is *haemophilus influenzae* and among viruses the *rhinovirus* [41]. About one quarter of all exacerbations are caused by coinfections with both a virus and bacteria, which result in an even worse outcome for the afflicted patient [42]. In about 20% of all exacerbations the trigger seemed to have been contact with environmental pollution [43]. During exacerbation pro-inflammatory cytokines, above all TNF-α, IL-6 and Il-8, are measurable in levels that even exceed the increase caused by the systemic inflammation in COPD. What follows is increased clustering and activation of neutrophils, which release ROS and proteases. This causes further damage of the airway epithelium and reduction in lung function [44]. Exacerbations have a huge impact on the survival of COPD patients. While 80% of patients without any exacerbations survive the next 5 years, only 30% of those with three or more exacerbations will live after 5 years. Among patients that needed to be admitted to a hospital because of the severity of the exacerbation, the survival rate sinks even further [45]. Due to the acute and massive inflammation of the airways, there is a 10% in-hospital mortality rate among patients with exacerbations of COPD [46]. On average, patients need between one week and 10 days to recover from an episode of exacerbation. One quarter of these patients will experience a relapse or a second event within the next month. Since microbes are the most common cause, prevention of infections are a focus of research.

2.2 Economic

As a global disease with millions affected, the economic burden connected to COPD are enormous. Estimations done by Mannino *et al*. in the year 2000 for the United States showed that this disease was responsible for 8 million physician office and hospital outpatient visits, 1.5 million emergency department visits, 726 000

hospitalizations and 119 000 deaths [47]. Other data from the US who evaluated the funds spent in 1993 on the estimated direct medical costs on COPD to be 15.5 billion US dollars [48]. Compared the total annual costs with other lung diseases COPD is only exceeded by respiratory cancer, surpassing asthma, influenza, pneumonia, and tuberculosis by billions.

Data collected in the European Union show that this disease is responsible for 6% of the total health care budget. Estimations that only look at expenses caused by respiratory diseases make COPD for 56% of the costs accountable [49]. Especially patients in later stages of the disease produce higher costs due to more medication, more hospital visits, more clinical tests, and the reduced ability to work (**Table 1**) [50]. Around 50% of the costs caused by COPD can be accounted to patients during exacerbations. Data from the UK show that exacerbations are with nearly 16% also one of the most common causes for admissions to an hospital and generate costs over 250 million pounds a year [51]. Mainly in developing countries the inability to work has a severe impact on the economy, while the costs for treatment and hospital stay are proportionally lower than in developing countries. Since studies predict that COPD will affect more people in the next decades, it has to be expected that the costs produced by this disease will rise accordingly. Most cost estimations don't take the effect of COPD on other diseases into account. The limitations in lung function make the treatment of conditions, especially those that rely on the mobility of the patient, more difficult.

Condition	Total Costs	Direct Medical Costs	Mortality	Morbidity	Total
COPD	23.9	14.7	4.5	4.7	9.2
Asthma	12.6	9.8	0.9	0.9	2.8
Influenza	14.6	1.4	0.1	13.1	13.2
Pneumonia	7.8	1.7	4.6	1.5	6.1
Tuberculosis	1.1	0.7	—	—	0.4
Respiratory cancer	25.1	5.1	17.1	2.9	20.0

*Adapted from the Division of Epidemiology, National Heart, Lung, and Blood Institute. Values given in billions of 1993 US dollars.

Table 1: Comparison of direct and indirect costs of lung diseases [50].

2.3 Risk Factors

According to the literature, tobacco smoking is the number one risk factor for the development of COPD [52]. Ezzati et al examined the mortality caused by smoking. In their presented data 4.83 million premature deaths can be linked to smoking worldwide in the year 2000. 970.000 thousand of those deaths were caused by COPD while the rest can be attributed to cardiovascular disease and lung cancer [53]. Other Factors with good evidence for an association with increased risk to develop COPD are outdoor air pollution, occupational exposures and alcohol intake [54]. Studies from Mexico and Columbia found that indoor pollution resulting from heating with biomass fuel and wood are linked to an increased risk for obstructive airway diseases [55, 56]. The effect of airborne particles at the workplace was investigated by Bakke et al. Workers with a low level of exposure to airborne particles had a significant lower risk of asthma and COPD than those with a high degree of exposure. Especially working places with exposure to quartz, metal gases and aluminum have a negative impact on airways [57].

2.4 Genetic Factors

As mentioned above, tobacco smoking is the major contributor for COPD next to a variety of other environmental factors. Additionally, genetics are a relevant factor for the occurrence of this disease, especially the fact that only one out of four smokers will develop COPD suggests that there has to be a genetic predisposition that makes a person more vulnerable to exogenous factors. A genetic disorder that is already known to be connected with COPD is alpha1-antitrypsin deficiency (AATD). Alpha1-antitrypsin is a serine protease inhibitor stopping enzymes like leukocyte elastase, proteinase-3 and cathepsin G released by inflammatory cells [58]. The production of AAT happens mainly in the liver and in macrophages located in alveoli. The first time a correlation with AATD and pulmonary emphysema was proposed was in 1964 by Eriksson, and shortly after a connection with liver cirrhosis was reported [59, 60]. The gene for AAT can be found on chromosome 14 and is encoded from the SERPINA1 gene. There are 4 different allele know that are named after their quickness in gel electrophorese.

In the healthy population the most common genotype is the normal homozygote MM allele. The homozygote ZZ allele is the most common in patients with severe AAT insufficiency. Although only 1-3% of all cases of COPD can be attributed to AATD, those patients suffer from a worse case of chronic bronchitis and obstruction harder and at an earlier age than patients without that genetic background.

Another study that tried to investigate genetic factors analyzed the development of COPD in twins. The results showed that monozygotic twins have a significant higher hazard ratio than dizygotic, with 4.3 in the Danish population and 3.4 in the Swedish [61].

2.5 Definition and Classification of COPD

COPD is a global disease that is preventable in most patients, and is treatable in others. COPD leads to a continual decline in airflow due to a chronic inflammation of the lung tissue caused by noxious particles like tobacco smoke. With the progression of COPD more areas of lung parenchyma and small airways are affected by the inflammatory process, which ends in a nonreversible destruction of lung tissue [62]. This decreases in functional lung tissue amplifies the trapping of air during expiration, which is seen as a major characteristics in the development of COPD [63]. Exacerbation of the disease further increases the morbidity and mortality of this disease [64]. According to the guidelines from the European Respiratory Society (ERS) and the Global Initative for Chronic Obstructive Lung Disease the gold standard to measure lung obstruction is spirometry [65-67]. The spirometric classification has proven to be cheap and widely available procedure to examine lung function and to predict the need for healthcare resources and mortality. These predictions are useful to evaluate populations but for the diagnosis of individual people spirometry is not sufficient.

Classification of Severity of Airflow Limitation in COPD

(Based on Post-Bronchodilatory FEV_1)

In patients with $FEV_1/FVC < 0.70$		
GOLD 1	Mild	$FEV_1 \geq 80\%$ predicted
GOLD 2	Moderate	$50\% \leq FEV_1 < 80\%$ predicted
GOLD 3	Severe	$30\% \leq FEV_1 < 50\%$ predicted
GOLD 4	Very Severe	$FEV_1 < 30\%$ predicted

In addition, a FEV1/forced vital capacity below 0.7 after the administration of a bronchodilator indicates of an airflow limitation that is not fully reversible.

In 2009 a British research group generated a short questionnaire that should help assess the quality of life of patients with COPD. This COPD Assessment Test (CAT) was validated on over 1500 patients in Europe and America. The patients test performance showed a significant correlation with the disease severity, making this test a simple and sensitive tool for monitoring COPD patients (**Figure 1**) [68].

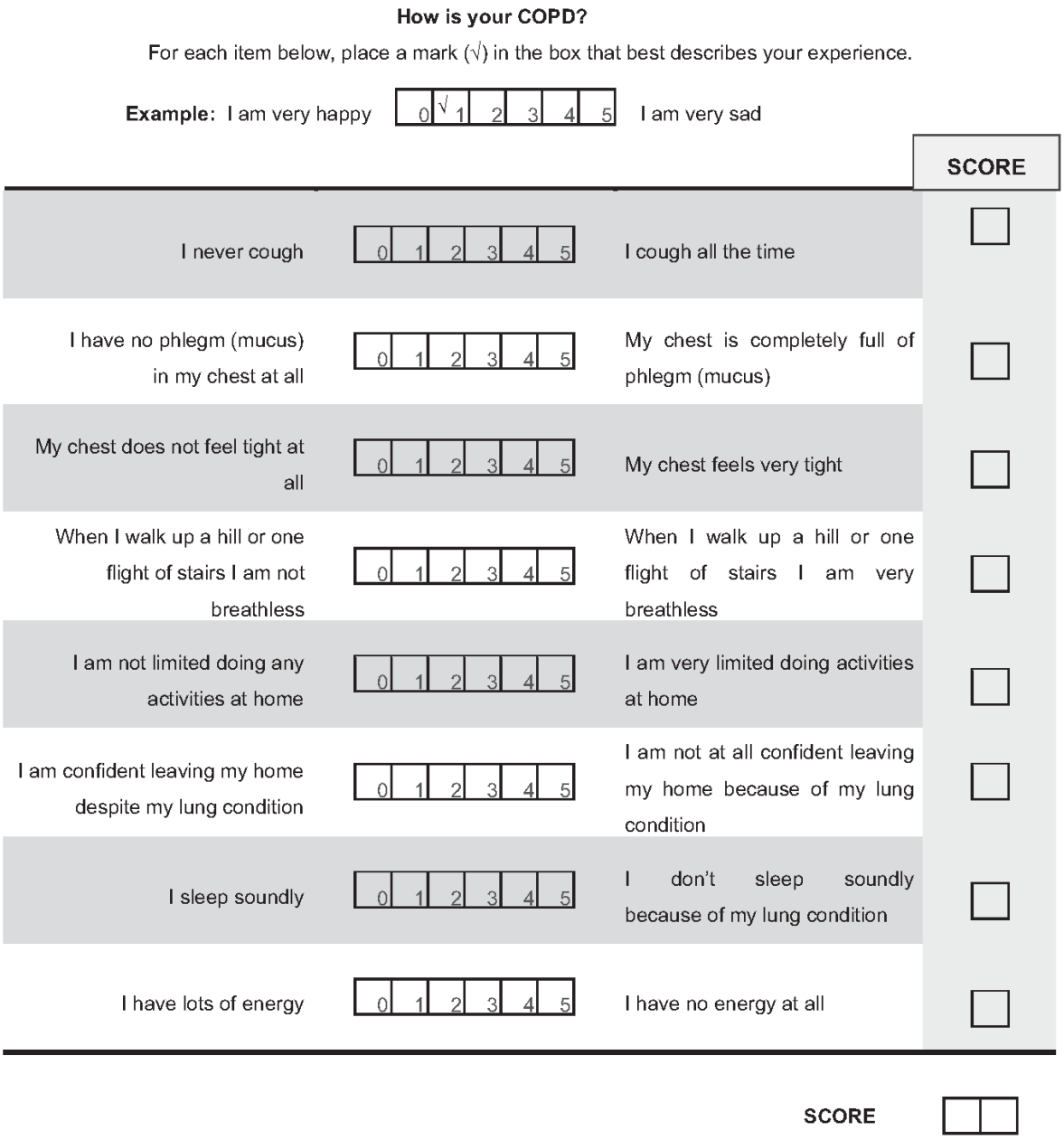

Figure 1: CAT questionnaire.

In addition the Medical Research Council generated a dyspnea scale that evaluates at what point the patient experiences breathlessness. In this questionnaire the patient is asked to choose the one statement that applies the best to his current condition, out of 5. These statements range from "not troubled with breathlessness except with strenuous exercise" to "too breathless to leave the house or breathless when dressing or undressing" [69].

Spirometry, questionnaires together with comprehensive assessment of patients' symptoms should lead to a valid diagnosis. Diagnosis should include a combined personalized evaluation, a classification of the severity of the disease, a forecast for the probability of exacerbations, an estimation of mortality and should initiate a suitable therapy.

2.6 Treatment of COPD

So far there is no cure to COPD, therefore the treatment of this disease focuses on the reduction of symptoms, preventing a disease progression, and on reducing the risk of exacerbations. Especially in the early stages of COPD the success of the treatment relies on the compliance of the patient. In order to minimize the risk of a progression of the disease all harmful noxa are removed. Since for most patients tobacco smoke was the cause for development of COPD, a complete Smoking cessation is crucial [70]. In some patients a medical support may be necessary in order to help with the withdrawal [71, 72].

Since air pollution is also an important risk factor, its exposure should be reduced as much as possible at the work place, indoors, as well as outdoors. It is recommended for COPD patients to perform daily physical activity, which is beneficial for the overall health [73, 74].

So far the pharmacologic therapy was not able to stop the long-term reduction of the lung function, but it helps with the symptoms, gives the patient a feeling of subjective well-being, and reduces the occurrence of exacerbations [75, 76].

According to the guidelines from the global initiative for chronic obstructive lung disease, patients can be divided into 4 groups going by their individual risk of exacerbation and the severity of their symptoms [77].

Patients with just mild symptoms and a low risk of exacerbation are placed in group A. This group of patients benefits most of short-acting bronchodilators [78]. Those substances work by dilating the bronchi by reducing the smooth muscle tone. The patients can easier exhale and the lung is less hyperinflated. This treatment can be combined with a long-acting bronchodilator, but so far there are no significant benefits for this method [79].

When the symptoms become more sever patients will be sorted into group B. In this group of patients long-acting bronchodilators are the right course of action. Which bronchodilator is used can be chosen individually as well as a combination of bronchodilators which can be prescribed [80]. Furthermore Theophyllin can be given, which also has the effect of relaxing the bronchial smooth muscle cells. This helps remove mucous from the lung via enhancing cilia movement, which reduces the pressure in the lung vessels and enforces the contractions of the muscles of respiration.

In group C, patients have just a few symptoms, but are a high risk for the occurrence of exacerbations. Patients in this group are usually COPD GOLD 3 or GOLD4, with intense airflow limitations. Here a treatment with long-acting bronchodilators and corticosteroids show the most benefits [80, 81]. The treatment with corticosteroids is controversial, patients experience relief of their symptoms and better lung function, but a withdrawal of this medication can raise the risk exacerbations further.

Patients that are in group D suffer from more than one episode of exacerbation a year in addition to having severe symptoms. The treatment consists of a combination of all the above mentioned medications. In addition drugs that help dissolve the mucous may be added [82, 83].

For some COPD patients other forms of treatment may be necessary. In cases of hypoxemia a long-term oxygen therapy is crucial. Patients with respiratory failure that receive this treatment display an improvement in survival rate [84].

2.6.1 Lung Transplantation

For a number of end-stage lung diseases, lung transplantation (LTx) is the last option. The most common pulmonary diseases that make an LTx necessary are COPD, idiopathic pulmonary fibrosis (IPF), peripheral pulmonary hypertension (PPH), cystic fibrosis (CF) and Alpha-1 antitrypsin deficiency. Since the introduction of LTx as a possible treatment, COPD is and always has been the diseases with the highest number of transplantations needed (**Figure 2**) [85].

A report that gathered data from 132 transplant centers in America states that with 33,5 percent COPD is the most common indication [86]. Data from the International Society for Heart and Lung Transplantation showed that worldwide in 2010 alone more than 3500 LTx were performed [87, 88].

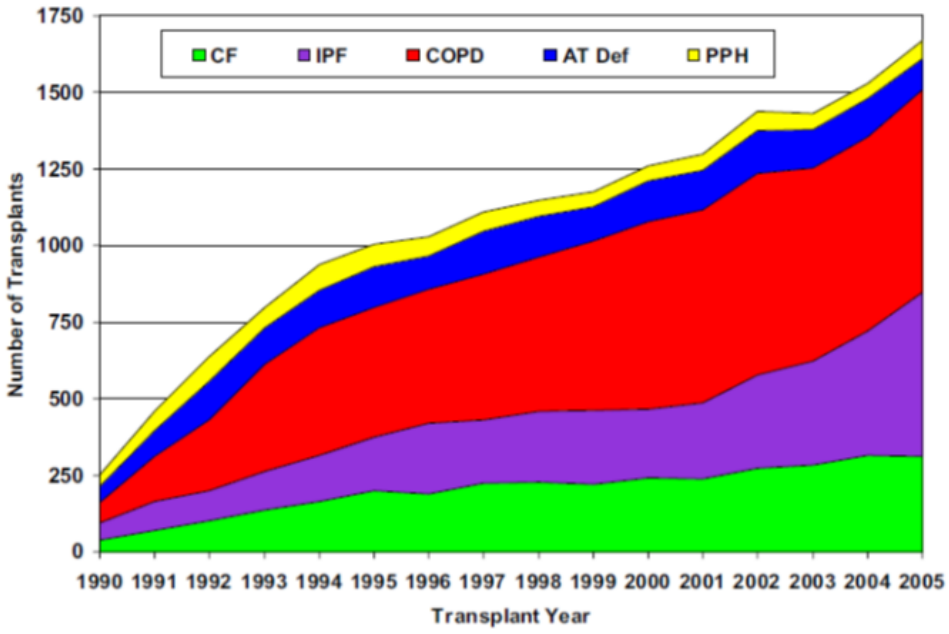

Figure 2: Indications for adult lung transplantations by year [85].

The conventional procedure is access via clamshell incision (**Figure 3**). Afterwards a dissection of the pulmonary hila follows, the pulmonary artery and the left atrium are clamped and finally the lung of the recipient is removed. What follows is the anastomosis of the bronchus and pulmonary artery of the donor organ. In order to prevent blood loss and entering of air into the left atrium, clamps, most commonly satinsky clamps, are applied.

Because of the complexity of operation and to reduce the risk for the patient, this procedure is normally performed under cardiopulmonary bypass [89]. At every point during the surgery the implantation of a cardiopulmonary bypass. Especially patient with PPH tend to not tolerate clamping of the pulmonary artery and end up hemodynamically unstable. Furthermore an insufficient gas exchange after unilateral dissection of the pulmonary hilum can make a CPB necessary [90].

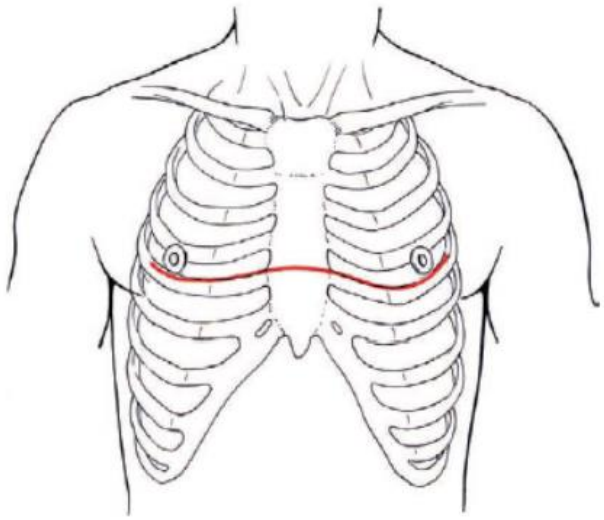

Figure 3: Clamshell-Thoracotomy [91]

2.6.2 Cardiopulmonary bypass

The cardiopulmonary bypass (CPB) was first successfully performed in 1952 by Forest Dewey Dodrill and soon became an important technic crucial in many operations like valve surgery, surgery on septal defects and operations concerning great vessels.

Extra corporeal circulation (ECC) made it possible to bypass the heart and gave the surgeon the option to arrest the heart by cardioplegia, without interrupting the blood supply to the head [92]. Cardioplegia provides a still, blood-free surgical field, which made it possible to achieve the precision and reproducibility necessary for direct coronary anastomosis. Parts of the CPB are the roller pump, which takes over the function of the heart, and the oxygenator, from which there are two types the capillary oxygenator and the membrane oxygenator.

In the oxygenator the venous blood gets resituated with oxygen and depleted of carbon dioxide. The capillary oxygenator works by carrying the blood through cavernous capillaries that are flushed with gas. The membrane oxygenator uses a semi-permeable membrane to separate blood from gas and relies on Fick's laws of diffusion to achieve the gas exchange. The latter type is less traumatising and can be used for several days, in case of impaired lung function. The usage of an extra corporal circulation makes it necessary to heparinise the blood in the heart-lung machine [93].

2.6.3 ECMO support

Although CPB has many advantages and is sometimes a necessary tool in cardio-thoracic surgery, it can also add a number of complications. Since the CPB uses a venous reservoir, a large dose of heparin is administered. In order to reduce bleeding the surgery is performed under low levels of blood flow (around 2.2 L/min/m^2) and low hematocrit levels (20%). The resulting decreased systemic oxygenation is controlled by induction of body hypothermia. As an alternative to CPB, extracorporeal membrane oxygenation (ECMO) can be used. It is a temporary support for patient with heart or lung diseases that are not able to provide sufficient oxygenation.

In contrast to CPB it only uses a partial cardiopulmonary bypass under physiological conditions with just a minimized use of heparin (**Table 2**) [1]. These properties make the ECMO perfect for supporting the patient during and after LTX.

	CPB	**ECMO**
Venous reservoir	Yes	No
Heparin	↑ Dose (>600 Units)	Titrated (120-180 Units)
Autotransfusion	Yes	No
Hypothermia	Yes	No
Hemolysis	Yes	No
Anemia	Yes	No
Arterial Filter	Yes	No

Table 2: CPB vs. ECMO [1].

Since ECMO became a technique that is widely available in comprehensive transplant centres, its use as a bridge before LTX became subject of research. Since the limited number of donor organs some patient need the ECMO support in order to survive until a fitting organ becomes available. Lang et.al investigated over the course of 13 years the survival of patients bridged on ECMO prior to LTX. On average these patients stayed 4.5 days on ECMO and although their worse initial situation they had a similar survival when compared to the normal LTX patients [94].

2.7 Pathogenesis

2.7.1 Innate Iummne System

COPD is defined as a progressive disease that affects part of the pulmonary system by decreasing the airflow generally triggered by airborne noxa. Additionally, there are also a number of systemic co-morbidities that are accompanied by COPD, that have a negatice effect on the survival of patients [3, 4]. COPD is characterized by an inflammation of the airways mediated by cells in the lung, above all epithelial cells and macrophages, which make up the vast majority of leukocytes in the pulmonary system [95]. Several authors were able to show neutrophils and macrophages in the bronchoalveolar lavage (BAL) of smokers, thus indicating an inflammatory response in the airways [96, 97]. In the tissue of COPD patient alveolar macrophages were present in a much higher amount than in controls, suggesting that in the state of chronic inflammation a high amount of immunoactive cells are recruited to the lung [98]. The activation of those cells leads to a release of chemotactic proteins, which attract further inflammatory cells into the tissue. This process is set into motion by a chronic stimulus, most of the time through cigarette smoke, and creates a self-amplifying state of inflammation. Even amongst smokers, only around 20% will end up with COPD, but in those patients even a cessation of smoking won't stop the chronic inflammation [99, 100]. Alveolar macrophages are seated on the surface of pulmonary alveolus, making them the first part of respiratory immunsystem between the respiratory tract and the air. Studies that examined the macrophages of COPD patients and healthy controls showed that those cells release a wide variety of pro-inflammatory cytokines like TNFα, IL-8, IL-1 [101]. Their ability to also produce matrix metalloproteinase (MMPs) is connected to the occurrence of emphysema in COPD. Through the chronic inflammation high levels of MMPs, especially MMP1, MMP9 and MMP12, a slow destruction of the extracellular matrix is induced [102, 103].

2.7.2 Adaptive Immune System

Although these findings propose an important role for the innate immunity in the occurrence of COPD in patients, several studies showed that the adaptive part of the immune system also plays a crucial role. Studies that counted the number of lymphocytes in the lung tissue and the airways found higher numbers of CD8+ lymphocytes in COPD patients when compared with healthy individuals. Further investigation showed that even in healthy smokers higher levels of lymphocytes were observed and that there was a correlation between the CD8+ cells and pack years [104]. When stimulated CD8+ lymphocytes have the ability of lysing targeted cells, by releasing proteins like perforin. Normally heightened levels of CD8+ cells are only observed in patients suffering from a viral infection, but in COPD patients those cells are found throughout the lung. Additionally, the numbers also correlate with severity of the disease [105-107]. Further analysis of CD8+ lymphocytes from COPD patients found heightened levels of the protein perforin in the cells when compared to those of healthy controls [108]. These findings suggest a link between CD8+ lymphocytes, the destruction of lung and airway tissue in COPD. Other studies that performed histological analysis of the airways and lung tissue of COPD patients showed high levels of CD4+ T-cells. Especially in emphysematous areas CD4+ cells were mainly found. Based on these observations it has been suggested that CD4+ T-cells are associated with pathological tissue remodeling [109-111]. Further analysis of these cells showed a high expression of the proteins IFN-γ, IP-10 and MIG which play an important role in the activation of macrophages [112].

2.7.3 Autoimmunity in COPD

In 2003 the hypothesis that COPD may have some similarities with an autoimmune disease was proposed. As mentioned above the chronic inflammation that is present in COPD patients leads to the activation and infiltration of immune cells into the lung parenchyma and the airways (**Figure 4**) [113].

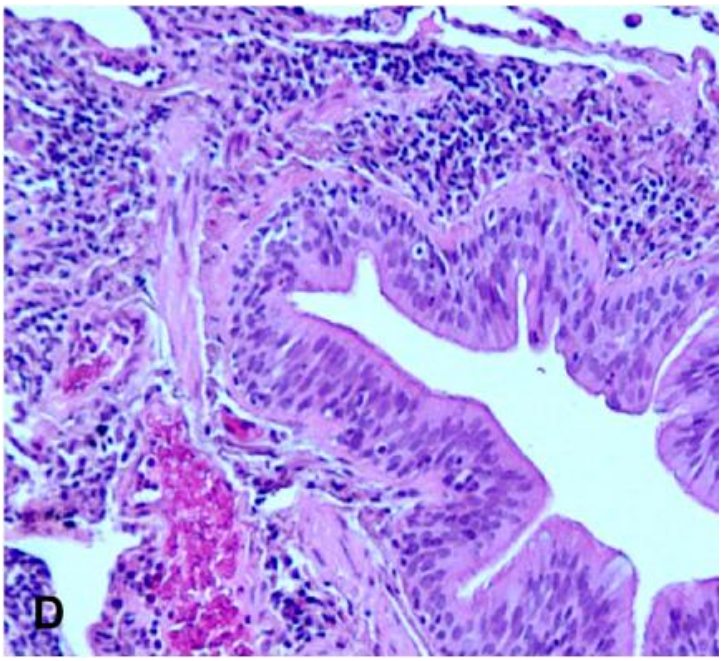

Figure 4: Lymphocytes infiltrating the adventitia in a bronchiole [113].

Once a patient reaches this stage, the process becomes self-perpetuating. Several studies that investigated the effect of tobacco smoking cessation in COPD patients found persistence of high numbers of lymphocytes in the lung [114-116]. Another interesting observation is that although there are many smokers, only a small amount of these people will develop COPD. This makes a genetic preposition probable, like it has been described in a number of autoimmune diseases [117-119]. Macrophages that were isolated out of the lung tissue of COPD patients showed to be lacking the ability to phagocytize dying or dead cells [[120]. The aggregation of apoptotic cells leads to a constant release of self-antigens. In the autoimmune disease systemic lupus erythematosus (SLE) is a crucial process for its development [121].

An important tool for the detection of autoimmune diseases is the search for autoantibodies, such as antinuclear antibodies (ANA) and anti-tissue (AT) antibodies [122]. A 2011 study showed that COPD patients have significantly higher levels of circulating ANA and AT compared healthy controls (**Figure 5**) [123].

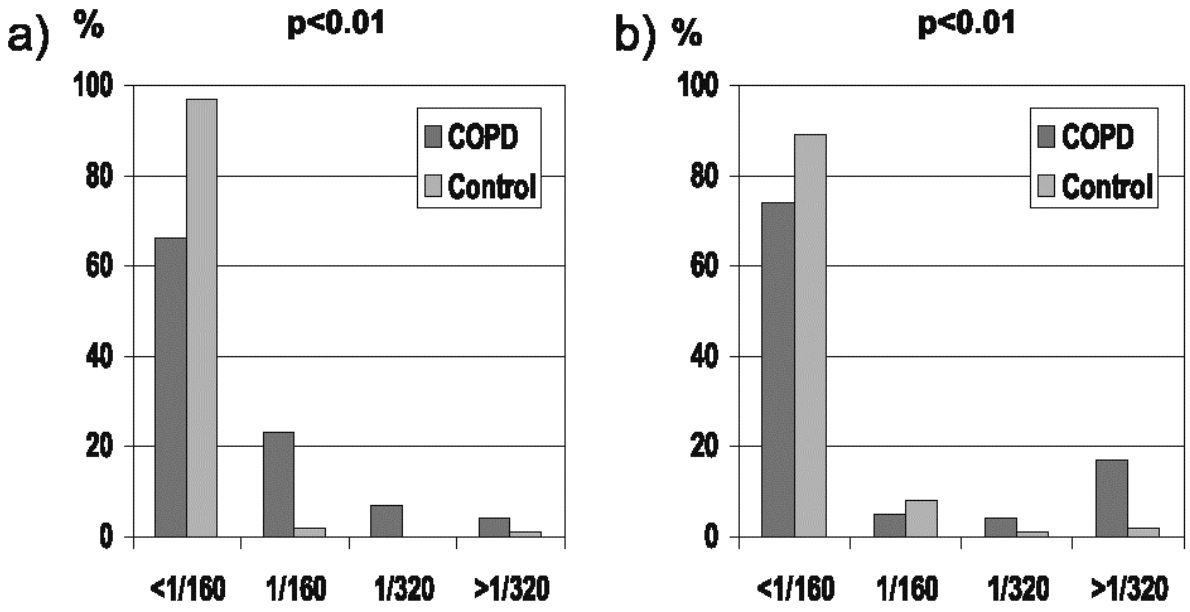

Figure 5: Frequency distribution of (a) antinuclear antibody (ANA) titers and (b) anti-tissue antibody (AT) titers in patients and controls [123].

2.8 CD4+CD28null cells

CD4+ T cells are an essential part of the adaptive immune system in the circulation of the healthy. Cd28 is a molecule that is commonly found on those cells and serves as a co-stimulator, interacting between the T cell receptor (TCR) and a peptide or the MHC complex. In healthy volunteers CD4+ cells that are missing CD28 on their surface, are found rarely in the circulation. However studies showed that in several autoimmune diseases like multiple sclerosis, Wegener's granulomatosis and ankylosing spondylitis, those cells can be found in high amounts [124-126]. The loss of the CD28 molecule is caused by the repeated antigenic stimulation that is happening in states of chronic inflammation and is also evidence for T-cell senescence [127].

Closer analysis of CD4+CD28null cells showed high levels of perforin and granzyme B, which are normally only detectable in cytotoxic T cells and Natural killer cell (NK cell) [128]. With the expression of those cytolytic proteins CD4+CD28null cells upon stimulation are able to lyse targeted cells and induce apoptosis.

However CD4+ T lymphocytes that are missing the CD28 expression show a dysregulation of the survival protein, bcl-2, which makes themselves resistant to certain apoptotic stimuli [129].

For T cell activation recognition and stimulation of an antigen is necessary which is presented by an antigen-presenting cell (APC). This process also requires interaction via a co-stimulatory receptor – normally the membrane bound CD28 molecule. CD4+CD28null cells are able to use different activation pathways to release the cytotoxic perforin. Through the release of Interferon- γ they are able to activate macrophages [130].

Another property CD4+CD28null cells have in common with NK cells is the expression of Killer immunoglobulin-like receptors (KIRs) on their cell surface [131]. KIRs normally help NK cells to recognise both tumor cells as well as cells infected with microorganisms as possible lysing targets. This subset of CD4+ cells also expresses Killer cell lectin-like receptor subfamily B, member 1 (KLBR1) on the cell membrane which is associated with a high expression of TNF-α and IFN-γ [132].

A close investigation of the surface of CD4+CD28null Cells reveals that they also express CD94, CD158 and CD161 receptors, which is also a characteristic of NK cells. All these findings suggest that these cells express features of both the adaptive and the innate immune system [133].

3. Previous Work

In 2008 Lambers et.al were able to describe elevated levels of CD4+CD28null cells in the peripheral blood flow of COPD patients when compared to gender and sex matched control groups. A high protein expression of perforin, granzyme B, and natural killer receptors were found in these cells. Stimulation of PBMCs separated from the blood of COPD patients with lymphocyte-specific anti-CD3 and PHA induced a high IFN- γ response when compared with healthy controls. Statistical analysis showed a significant negative correlation between the amount of circulating CD4+ cells lacking CD28 and the patients performance in spirometric evaluations. In a subgroup of smokers, measurement of CD4+CD28null cells showed the ability to be used as a prediction marker to diagnose COPD (**Figure 6**) [134].

These results suggest an important role of CD4+CD28null cells in the pathogenesis of COPD.

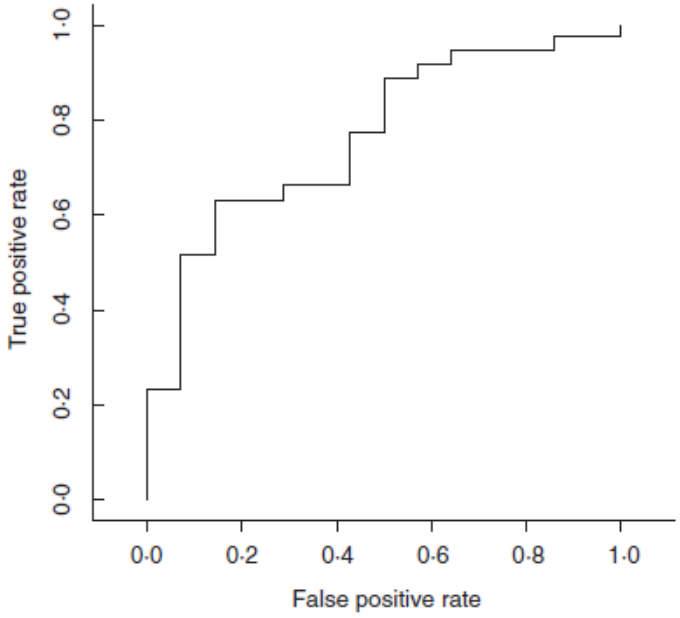

Figure 6: The prediction capacity of CD4+CD28null cells in COPD showed in a logistic regression analysis. ROC curve analysis revealed an AUC of 0,76 [134].

4. Rationale and Aim of the Study

CD4+CD28null cells seem to be a crucial part in the pathogenesis and progression of autoimmune disease, chronic inflammation, and tissue damage. CD4+CD28null cells were shown to be systemically heightened in COPD and can be found in large numbers in explanted COPD lungs. We aimed to further define their role in the damaged lungs of endstage COPD patients (GOLD IV). For these prupose we analysed homogenized tissue samples from explanted COPD lungs obtained from patients undergoing Lung transplantation.

We performed stimulation experiments with CD4+ cells, purified from COPD tissue samples. The proliferative capacity of CD4+CD28null cells stimulated with elastin peptides was crucial to assess the auto-reactive properties of these cells.

5. Materials and Methods

5.1 Proband Selection

We enrolled a total of 18 subjects to this study. 13 consecutive patients suffering from COPD receiving a donor organ were included.

Inclusion criteria:

proven diagnosis of COPD and IPF according to the Consensus Report From the Pulmonary Scientific Council of the International Society for Heart and Lung Transplantation [135]

single or double lung transplantation

age >18

written informed consent

Exclusion criteria

treatment with blood transfusions during the last 8 weeks

other autoimmune disorders or diseases shown to have systemically increased numbers of CD4CD28null (rheumatoid arthritis, Wegener granulomatosis, acute coronary syndrome)

similar participation of another study

5 lung samples gathered from patients undergoing lung resection served as controls.

Inclusion criteria:

No evidence for COPD nor any form of autoimmune disease

age >18

written informed consent

Groups will be age matched (+/- 2.5 years) and gender matched. COPD and IPF patients will be recruited from the Dept. of Cardiothoracic Surgery, Medical University of Vienna.

5.2 Sample Size Calculation and Study Durability

The main criteria of our study will be the elastin specific proliferative capacity of CD4CD28null cells obtained from COPD lung tissue samples. To the best of our knowledge, no previously performed studies exist addressing the evaluation of infiltrating CD4CD28null cells in pulmonary diseased patients. The only study addressing autoreactivity in CD4+ cells was published by Lee et al [136]. Based on their findings we assume that 50% of CD4CD28null cells from COPD lungs will to a certain extent show an anti-elastin reactivity. In contrast to that CD4CD28null cells from control lungs should show a reactivity agains elastin in less that 10 percent of all cases.

5.3 Methods

The study protocol was approved by the ethics committee of the Medical University of Vienna (EK no. 1113/2009), and was performed in accordance with the Declaration of Helsinki. Thirteen end-stage COPD patients, who were transplanted at the Department of Thoracic Surgery, Medical University of Vienna, participated in the study. For controls, age- and gender-matched nonCOPD patients, who were operated at our department for earlystage primary lung cancer (n = 4) or spontaneous pneumothorax (n = 1) served as controls. A detailed patients' demographic is depicted in Table 1. Whole blood samples were drawn before the operation by venipuncture and samples were further processed immediately thereafter.

5.4 Tissue homogenization

Peripheral lung specimens of 2 × 2 × 2 cm were collected from explanted COPD lungs or resected nonCOPD lung segments in the operation theatre immediately after explantation in order to preserve high tissue quality. In case of bilateral transplantation only one lung was evaluated (randomly chosen).

Since small airways have previously been described as the major structure with CD4+ cellular infiltrates, we chose to take tissue samples from the lung periphery.

To avoid bias due to selective sampling, six samples were taken following a standard procedure: Two samples were excised from the inferior lobe, two from the middle lobe/lingual and two from the superior lobe, respectively. Samples were processed immediately in order to avoid loss of viability. The tissue samples were shredded and single cell suspensions were produced by passing the tissue through 70 and 40 µm cell strainers (BD, NJ, USA). Homogenates were processed by Ficoll density gradient centrifugation and the mononuclear cell fraction was further purified by CD4+ Dynal magnetic beads (Invitrogen, CA, USA) following the manufacturer's instruction. After removing the labeling beads untouched, CD4+ T cells were recovered. Purity obtained was above 95 % as determined by flow cytometry. Proliferative response was measure by 3H-thymidine incorporation (18hrs) after 5 days.

5.5 Flow cytometry

Blood samples (after lysing red blood cells with a commercially available lysing buffer, Sigma-Aldrich, MO, USA) and tissue cell suspension were stained with fluorescein isothiocyanate (FITC)-conjugated antiCD4 and electron coupled dye (ECD)-conjugated antiCD28 or corresponding isotypes (both (Beckman Coulter, CA, USA) for 30 min). Cells were washed and 2×10^5 cells were analyzed for their content of CD4+CD28null cells on a Coulter flow cytometer (FC500, Coulter, CA, USA). Percentages of CD4+CD28null cells refer to the total CD4+ cell population.

5.6 Proliferation experiments

1×10^5 CD4+ cells, purified from lung tissue of four different COPD patients were incubated with irradiated allogeneic peripheral blood mononuclear cells (PBMC) in the presence or absence of human lung elastin peptides (prepared by enzymatic hydrolysis of human lung elastin), solubilized lung elastin (by successive extractions with hot oxalic acid), and human lung collagen type I (all Elastin Products Company, MO, USA; 30 ng/mL). The addition of IL-2 (BD, NJ, USA; 0.6 U/mL) to the experimental setting served as positive control. Plates were incubated for 5 days and

then pulsed for 18 h with 3H-thymidine. Proliferation was measured in a liquid scintillation counter.

5.7 Statistical analysis

Results are depicted as means ± standard error of the mean, and levels of significance were determined by Mann–Whitney test. Data analysis was performed with SPSS 18.0 (SPSS inc., United States) and GraphPad Prism 5 (GraphPad Software Inc., California, USA). A p-value less than 0.05 was regarded as statistically significant.

5.8 Research Facility

All the laboratory work will be performed at the Department of Surgery (surgical research facilities), Medical University Vienna.

5.9 Funds

Surgical Research Laboratories, Medical University Vienna and the Christian Doppler Research Association.

6. Ethical and legal aspects

6.1 Risk/benefit ratio

The expected risk for all probands involved in this study can be considered minimal. A single blood draw of 25 ml from all probands will be sufficient. For cytotoxicity assays, a second blood draw of 10 ml will be needed from selected patients. Patients admitted to the Department of Pulmonary Medicine will undergo routine diagnostic procedures including spirometry. Healthy volunteers will be routinely clinically examined to evaluate crucial parameters needed for statistical comparison such as FEV_1 and age.

With this study, we hope to describe a possible pathway in the pathogenesis of COPD, aiming at the long-term establishment of an effective causal treatment.

6.2 Legal aspects

The study was conducted in accordance with the guidelines of Helsinki (1964). A positive vote of the ethic commission was necessary before the study is initiated (EK no. 1113/2009).

Every proband has given his/her written consent of approval before taking part in the study. Aim of the experiments and risks of this study as well as clinical procedures, e.g. spirometry, were explained in detail to every participant before enrolment.

Samples were coded with numbers from 1 to 18. Probes were destroyed after analysis. Data obtained in this study was and will be treated confidentially. Names of participants were not published. Data collected from this study will be stored locked. All investigators are bound to the professional discretion of physicians.

7. RESULTS

7.1 Demographical Data

	Gender	Age	FEV_1%	FEV_1% VC_{max}	TLC	Medication	Smoker/PY
Patient 1	M	52	29	55	131	Th, ACH, BA, INH-C	Yes/25
Patient 2	M	49	28	39	189	Th, ACH, BA, INH-C	Yes/65
Patient 3	F	65	30	52	141	Th, ACH, BA, INH-C, syst-C	Yes/40
Patient 4	F	57	31	48	160	Th, ACH, BA, INH-C	Yes/30
Patient 5	F	58	15	57	107	Th, ACH, BA, INH-C, syst-C	Yes/38
Patient 6	F	62	13	50	171	Th, ACH, BA, INH-C	Yes/37
Patient 7	F	55	28	62	131	Th, ACH, BA, INH-C, syst-C	Yes/35
Patient 8	F	48	14	47	129	Th, ACH, INH-C, syst-C	Yes/30
Patient 9	M	63	15	36	160	ACH, BA, INH-C	Yes/40
Patient 10	M	58	23	34	132	ACH, BA, INH-C	Yes/100
Patient 11	M	59	23	37	130	ACH, BA, INH-C, syst-C	Yes/50
Patient 12	F	54	17	43	148	Th, ACH, BA, INH-C	Yes/90
Patient 13	F	39	36	51	125	Th, ACH, BA, INH-C, syst-C	No
Control 1	M	61	71	83	120	BA	Yes/80
Control 2	M	87	75	87	109	/	Yes/75
Control 3	M	18	Spontaneous pneumothorax—no lung function available			/	No
Control 4	F	79	61	81	111	/	No
Control 5	M	48	95	93	115	/	No

Table 3

Patients' characteristics are depicted in **Table 3**. COPD patients and healthy controls were well matched in terms of gender, age, and smoking habits. Participants from the COPD group were all in Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV suffering from a severe airway obstruction. (*Th* theophylline, *ACH* anticholinergic drugs, *BA* beta-2-adrenergic agonist, *INH-C* inhalative cortisone, *syst-C* systemic cortisone, *FEV* forced expiratory volume, *TLC* total lung capacity)

7.2 CD4+ purity

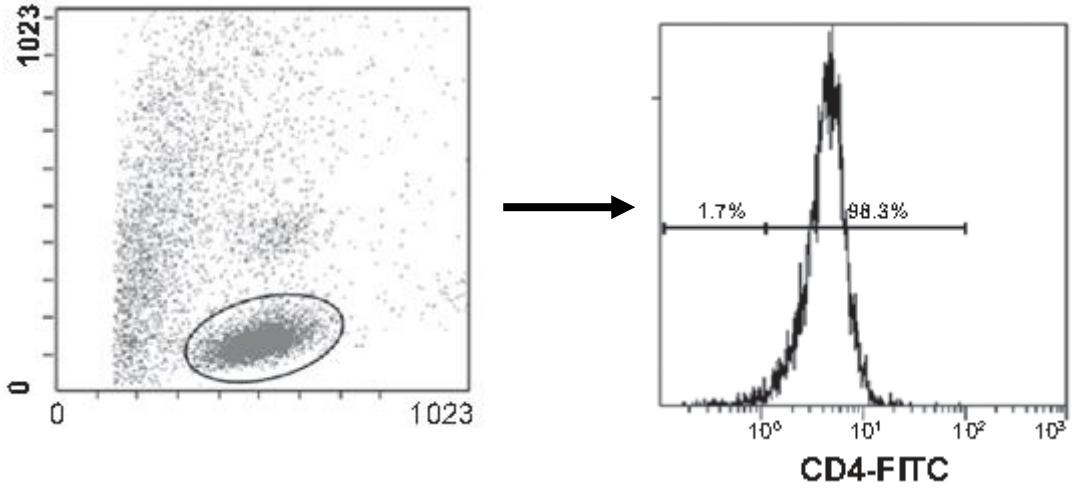

Figure 6: A representative case to illustrate the purity of CD4+ cells after separation from lung tissue homogenates. More than 95 % of the cells were CD4 positive as determined by flow cytometry.

7.3 CD4+ cells control vs. COPD

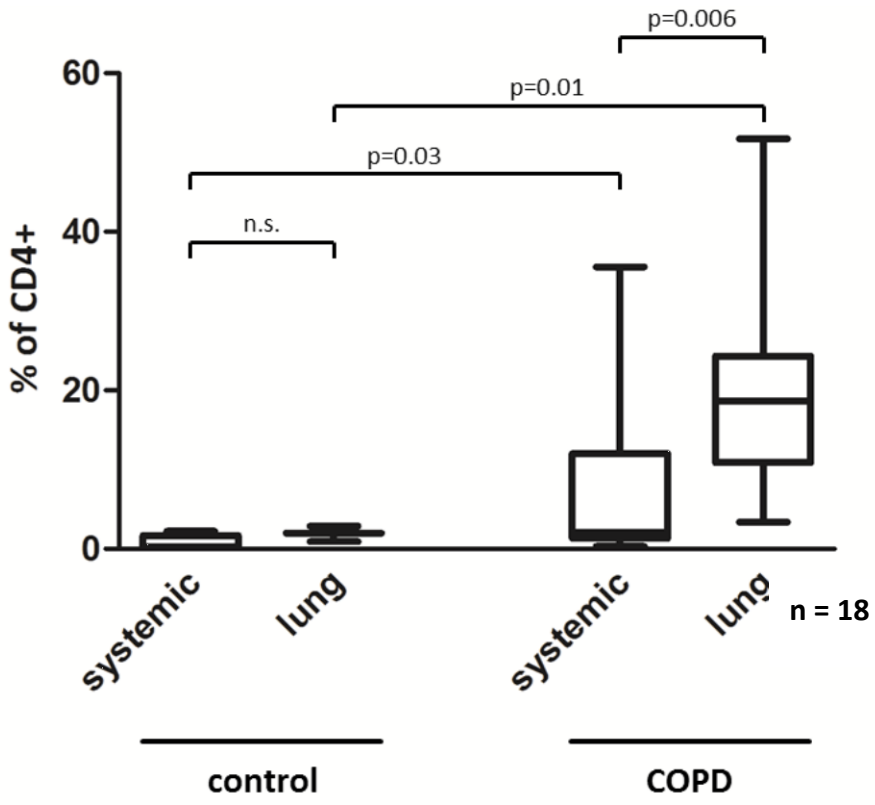

Figure 7: Percentages of CD4+CD28null cells of all CD4+ cells in whole blood samples and lung homogenates. Increased numbers of circulating CD4+CD28null cells were present in all COPD patients with a mean ± SEM of 8.02 ± 2.9 %. Healthy age-matched controls had only marginal numbers of CD4+ cells lacking CD28 in their circulation 0.79 ± 0.4 % (n= 18).

7.4 CD4+ cells systemic vs. lung

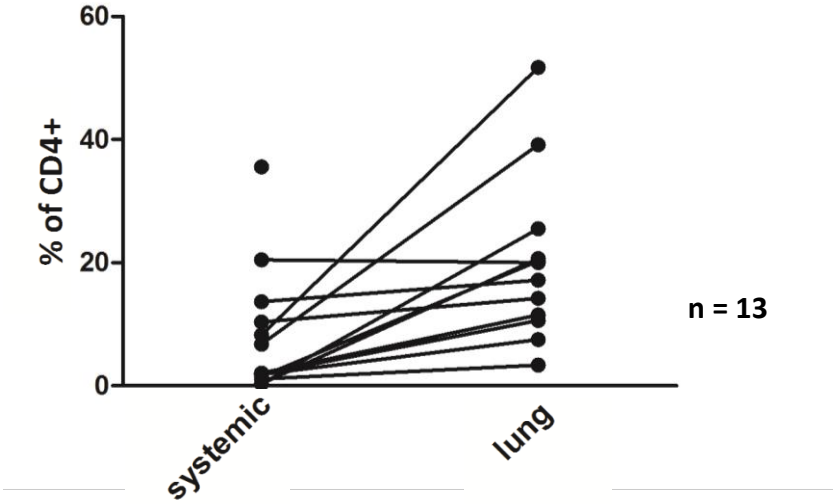

Figure 8: Every single COPD patient evidenced an increase of CD4+CD28null cells in the lung tissue homogenates when compared with systemic levels 20.15 ± 3.9 %; p = 0.006 (n=13).

7.5 Representative FACS analysis of a COPD patient

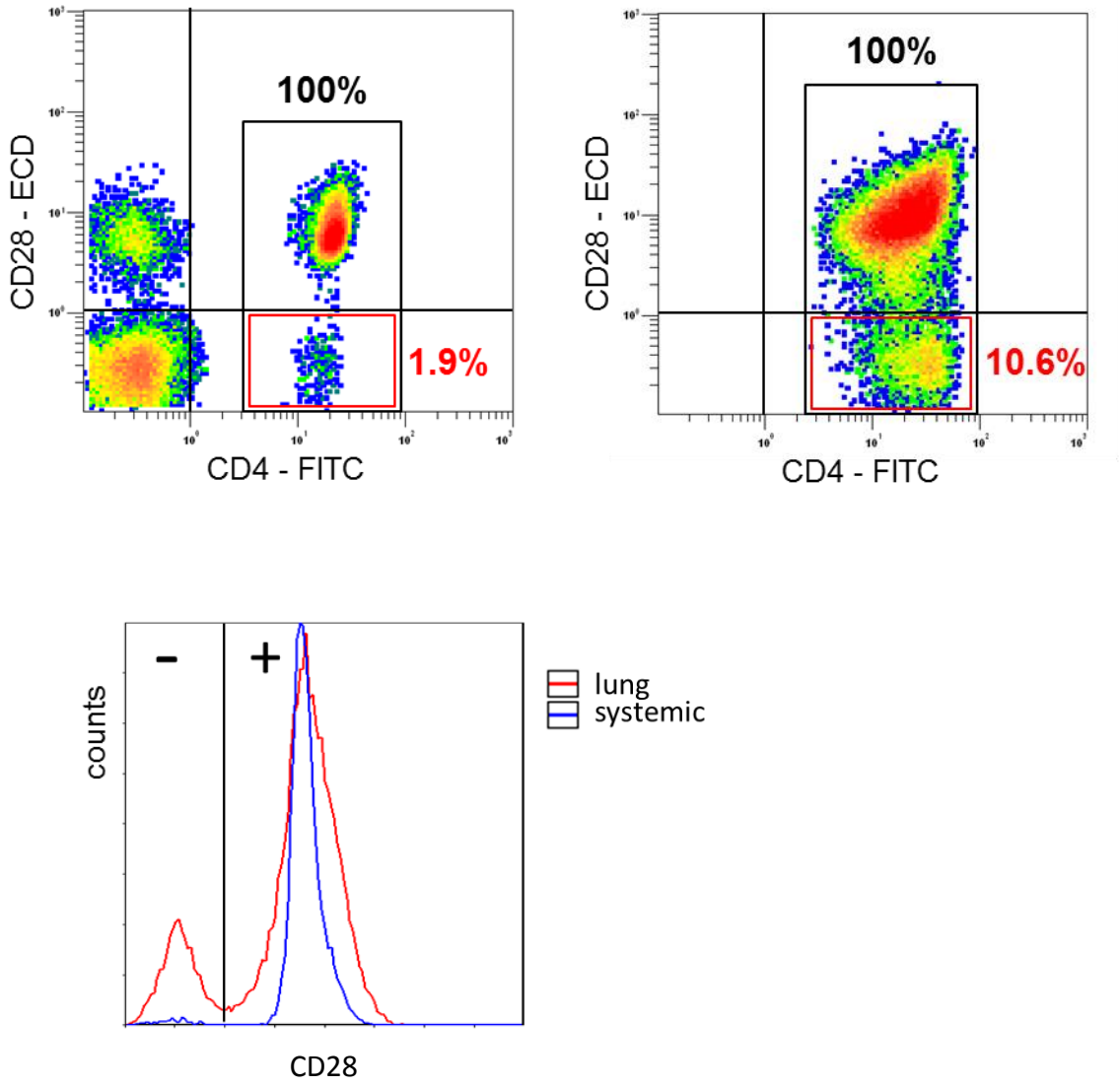

Figure 9: A representative case of a COPD patient. While only 1.9 % of the measured CD4+ cells were lacking the co-stimulatory CD28 molecule in the circulation, 10.6 % showed that trait in the lung tissue. Underneath depicted in form of a histogram.

7.6 CD4+ proliferative response

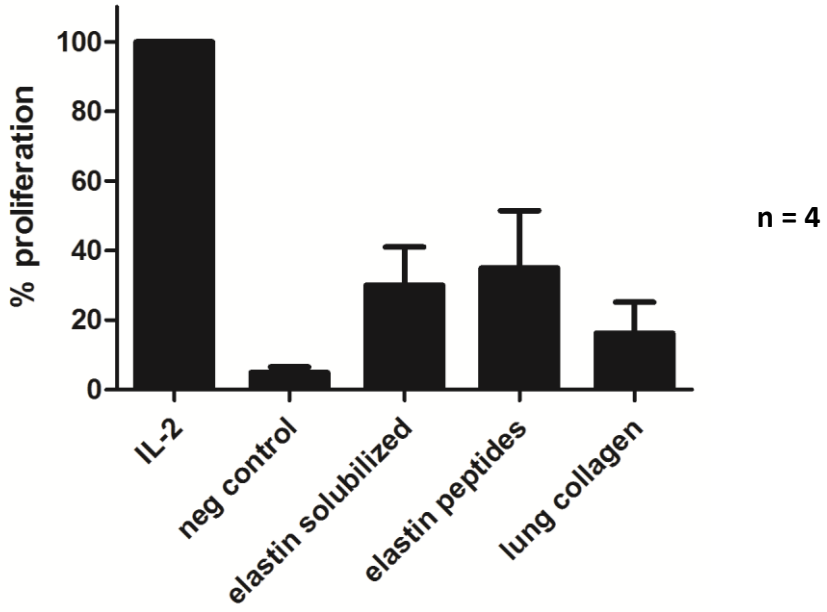

Figure 10: Proliferative response of lung resident CD4+ cells. Coincubation of CD4+ T cells with human lung collagen type I, human lung elastin peptides, and solubilized elastin, led to a stable proliferation of lung resident CD4+ T cells (n = 4). However, this observation did not lead to levels of significance due to the relatively small sample size.

8. Discussion

With this work we were able to show CD4+CD28null cells are not only detectable in the circulation, but also in the lung parenchyma. When compared to lung tissue from control patients, COPD patients in stage IV showed high amount of CD4+ that was missing the CD28 co-stimulatory molecule. In addition, when this sub-population of CD4+ cells were co-incubated with components of the extracellular matrix, these cells react with proliferation. A direct involvement of CD4+CD28null cells in the pathomechanism of COPD was still in debate. This is because so far there was no evidence that these cells are resident in the lungs of patients with chronic pulmonary obstruction. Since CD4+ T cells are a key part of the adaptive immune system, an involvement in the pathomechanism of COPD seems natural.

Previous studies that performed histological analysis of lung parenchyma gathered from COPD patients were able to find peribronchal CD4+ T cells in high numbers. These findings correlated with the severity of the airway obstruction [109, 137, 138]. Despite the fact that the clustering of CD4+ cells in the lung tissue is already described as part of the COPD pathology, there are no references about the effect of CD4+CD28null cells that are located in the lung parenchyma. The missing CD28 membrane molecule seems to be the result of the chronic stimulation that happens in inflamed airways [129]. In a number of autoimmune diseases, like Rheumatoid arthritis, Wegener's granulomatosis, Ankylosing spondylitis, Multiple sclerosis and Inflammatory bowel disease, high amounts of circulating CD4+CD28null cells have already been described [139]. Several properties of these cells, like their autoreactivity and the fact that they are clonally expanded, make it probable that they are responsible for the autoimmune response.

A previous study was able to find elevated levels of CD4+CD28null cells in peripheral blood of COPD patients. Not only did the COPD patients have significant higher levels, but also their levels correlate with the severity of the disease [134]. Another research group found similar results, however, they were not able to find a significant difference between healthy controls, smokers, and COPD patients. However, this could be due to the fact that they only included low to moderate stages of COPD and did not separate them in their analysis [140]. So far there is no proof that CD4+CD28null cells are involved in either the development, nor the maintenance, of the chronic inflammation and degeneration of lung tissue. Our aim was to find

evidence for a link between these cells and COPD. In order to do so, we gathered lung tissue specimen from patients with COPD stage IV and from patients with no indications for a chronic lung disease. By performing flow cytometric analyses on the homogenized lung tissue we were able to find significantly higher counts of CD4+CD28null cells than in the peripheral blood. Furthermore those cells were completely missing in the lungs of our healthy controls. These findings suggest that CD4+CD28null cells that are resident in the lung play a role in the pathomechanism of COPD.

A study from 1997 was able to find a high expression of IFN-γ in these cells [141]. The release of IFN-γ leads to an activation of macrophages which react with the secretion of MMPs. Through the constant degeneration of extracellular matrix caused by MMPs the development of emphysema is induced. Nakajima et al. described that CD4+CD28null cells are able to target endothelial cells directly and cause cell lysis [128]. Based on these findings we wanted to evaluate the autoreactive abilities of these cells. We were able to purify CD4+ T cells out of the lung specimens. When we incubated the cells with lung specific elements of the extracellular matrix, a proliferative response was measurable. Due to the small sample size this result did not reach levels of statistical significance. Nevertheless this is the first time that a group was able to gather and purify lung resident CD4+ T cells from different patients, in a high enough concentration in order to analyze their proliferative capacity.

Autoreactive cells targeting airway epithelium and lung parenchyma represented a crucial part of the hypothesis from when COPD was first described to have characteristics of an autoimmune disease. A study, that investigated autoimmune pathology induced by tobacco smoking, was able to detect anti-elastin autoimmunity in the circulation of patients suffering from end-stage COPD. In addition they were able to separate CD4+ T cells out of the peripheral blood and stimulated them with elastin peptides. Only the supernatant from the cells harvested from COPD patients showed a response, by releasing of high amounts of IFN-γ. When they evaluated their autoreactivity against collagen type I, they were not able to detect a response [136]. This finding may be attributed to the fact that their experiments were not performed on lung resident T cells.

With this work we are able to present evidence to support the hypothesis that COPD is characteristics of an autoimmune disease. Further investigations are necessary to elucidate the clonality of CD4+ cells with the characterized loss of co-stimulatory CD28. Also a systematic epitope mapping would help to filter out the autoantigens that induce the destruction of lung tissue in COPD. Our findings further corroborate CD4+CD28null cells a key factor in the chronic inflammation in COPD. Therapeutic approaches that target the function of CD4+ cells might be a new option in the treatment of COPD.

9. Abbreviations

AATD	Alpha 1-antitrypsin deficiency
ACH	Anticholinergic drugs
ANA	Antinuclear antibodies
APC	Antigen-presenting cell
AUC	Area under the curve
AT	Anti-tissue
BA	Beta-2-adrenergic agonist
BAL	Bronchoalveolar lavage
CAT	COPD Assessment Test
CD	Cluster of differentiation
CF	Cystic fibrosis
COPD	Chronic Obstructive Pulmonary Disease
CPB	Cardiopulmonary bypass
CRP	C-reactive protein
ECC	Extra corporeal circulation
ECMO	Extracorporeal membrane oxygenation
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence activated cell sorting
F	Female
FEV_1	Forced Expiratory Volume in 1 second
FVC	Forced vital capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
IFN	Interferon
IL	Interleukin
INH-C	Inhalative cortisone
IPF	Idiopathic pulmonary fibrosis
KIRs	Killer immunoglobulin-like receptors

KLBR1	Killer cell lectin-like receptor subfamily B, member
LTX	Lung transplantation
M	Male
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
NK cell	Natural killer cell 1
PHA	Phytohaemagglutinin
PPH	Peripheral pulmonary hypertension
PY	Pack years
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
Syst-C	Systemic cortisone
TCR	T cell receptor
Th	Theophylline
TLC	Total lung capacity
TNF	Tumor necrosis factor

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11. Appendix – Published Paper

original article

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High levels of lung resident CD4+CD28null cells in COPD: implications of autoimmunity

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Hohe Zahl Gewebs-infiltrierender CD4+CD28null Zellen in COPD-Lungen

Zusammenfassung Die chronisch obstruktive Lungenerkrankung (COPD) zählt zu den häufigsten Todesursachen weltweit. Die der Erkrankung zu Grunde liegenden, pathogenetischen Mechanismen sind noch immer großteils unbekannt. Allerdings zählt eine kontinuierliche toxische Schädigung durch Rauchen, welche letztlich zu einer sich selbst-erhaltenden, entzündliche Reaktion führt, als pathophysiologischer Schlüsselfaktor. Hinweise, dass Autoimmunität beim Krankheitsprozess eine Rolle spielen könnte, haben kürzlich großes Aufsehen erregt.

Während der chronischen Phase einer Autoimmunreaktion verlieren Lymphozyten ihre kostimulatorischen Signale. In früheren Studien konnte eine erhöhte Zahl von CD4+CD28null Zellen in der Zirkulation von COPD Patienten nachgewiesen werden. Allerdings ist eine direkte Beteiligung dieser CD4+CD28null Zellen an der Pathogenese der COPD umstritten, da es bis dato keinen Nachweis gibt, ob CD4+CD28null Zellen unmittelbar in der Lunge erkrankter Patienten entstehen. Im Rahmen dieser Studie wurden Lunge von Stadium IV COPD Patienten untersucht und die Zahl von Lungen-infiltrierenden CD4+CD28null Zellen mit der Anzahl an zirkulierenden CD4+CD28null Zellen verglichen.

Es konnte gezeigt werden, dass CD4+CD28null Zellen in hoher Konzentration im Lungengewebe von COPD Patienten vorhanden sind. Dies lässt auf eine direkte Beteiligung dieser Zellen an der Pathophysiologie der COPD schließen. Zusätzlich zeigten aus dem Lungengewebe aufgereinigte CD4+ Zellen eine stabile proliferative Antwort auf lungenspezifisches Elastin und Kollagen.

Diese Ergebnisse unterstützen die Autoimmunhypothese und die Rolle von autoreaktiven CD4+ Zellen in der COPD. Eine Modulation der CD4+ Zellfunktion könnte zukünftig therapeutisch genutzt werden.

Schlüsselwörter: COPD, Autoimmunität, chronische Entzündung, CD4+CD28null

Summary Chronic obstructive pulmonary disease (COPD) is a worldwide burden and a major cause of death. Pathogenetic mechanisms underlying the disease are still largely unknown. However, a continuous toxic injury due to tobacco smoking leading to a self-maintaining inflammatory process is considered a key factor in the pathophysiology of the disease. Evidence that autoimmunity might be involved in the maintenance of COPD has been recently noticed with great interest.

During the chronic phase of an autoimmune response, lymphocytes lose their costimulatory signals. Previously, CD4+CD28null cells were reported to be systemically heightened in COPD patients. However, a direct role of CD4+CD28null cells in the pathogenesis of COPD is still under discussion, since there is no evidence that CD4+CD28null cells originate from the lungs of diseased patients. Therefore, we evaluated lungs from end-stage COPD patients and compared the levels of tissue infiltrating CD4+CD28null cells to systemic levels.

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We could show that CD4+CD28null cells are present in high amounts in lung tissue obtained from COPD GOLD IV patients suggesting a direct involvement of those cells in the pathophysiology of COPD. Furthermore, purified lung-resident CD4+ cells showed a stable proliferative response to lung specific elastin and collagen.

These results further corroborate the role of autoreactive CD4+ cells in the maintenance of the inflammatory destruction in COPD. Modulating CD4+ cell function might be a new promising tool for future therapeutic approaches.

Keywords: COPD, auto immunity, chronic inflammation, CD4+CD28null

Introduction

Chronic obstructive pulmonary disease (COPD) is one of the leading health issues worldwide. The disease is characterized by a progressive decline of the expiratory airflow. Chronic inflammation of the lung tissue is thought to be one of the key features in the pathogenesis of COPD [1–3]. For decades, components of the innate immunity were considered pivotal in the development of emphysema [4]. However, recent reports demonstrated increased CD4+ lymphocytes—as part of the adaptive immune response—in bronchoalveolar lavage and tissue samples of COPD patients [5, 6].

The idea of COPD being a disease with an autoimmune component emerged some years ago [7]. The autoimmune hypothesis has been corroborated by Lee et al. [8] showing that CD4+ T cells from peripheral blood of COPD patients released interferon (IFN)-γ and interleukin (IL)-10 in response to elastin peptides.

Our group has previously shown that a special subpopulation of CD4+ cells, lacking their costimulatory surface marker CD28, is heightened in the circulation of COPD patients [9]. This unique cell population was increased dependent on disease stage when compared to age and sex-matched healthy smokers and non-smoking controls. CD4+CD28null cells are thought to be involved in the pathogenesis of several autoimmune diseases [10].They express high levels of intracellular perforin and granzyme B, enabling them to lyse target cells upon stimulation [11]. On the other hand, CD4+CD28null cells have been identified to be highly resistant against proapoptotic signals leading to their persistence in chronic inflammatory processes [12]. In contrast to our findings, Hodge et al. [13] have recently only found a weak correlation between circulating CD4+CD28null cells and COPD.

Since the impact of circulating CD4+CD28null cells in COPD is still unclear, we sought to further highlight their role in the pathophysiology of the disease by analyzing tissue samples of explanted end-stage COPD lungs obtained during lung transplantation.

Methods

The study protocol was approved by the ethics committee of the Medical University of Vienna (EK no. 091/2006), and was performed in accordance with the Declaration of Helsinki. Thirteen end-stage COPD patients, who were transplanted at the Department of Thoracic Surgery, Medical University of Vienna, participated in the study. For controls, age- and gender-matched nonCOPD patients, who were operated at our department for early-stage primary lung cancer ($n=4$) or spontaneous pneumothorax ($n=1$) served as controls. A detailed patients' demographic is depicted in Table 1. Whole blood samples were drawn before the operation by venipuncture and samples were further processed immediately thereafter.

Tissue homogenization

Peripheral lung specimens of 2 × 2 × 2 cm were collected from explanted COPD lungs or resected nonCOPD lung segments in the operation theater. Samples were shredded and single cell suspensions were produced by passing the tissue through 70 and 40 µm cell strainers (BD, NJ, USA). Homogenates were processed by Ficoll density gradient centrifugation and the mononuclear cell fraction was further purified by CD4+ Dynal magnetic beads (Invitrogen, CA, USA) following the manufacturer's instruction. After removing the labeling beads untouched, CD4+ T cells were recovered. Purity obtained was above 95 % as determined by flow cytometry (Fig. 1a).

Flow cytometry

Blood samples (after lysing red blood cells with a commercially available lysing buffer, Sigma-Aldrich, MO, USA) and tissue cell suspension were stained with fluorescein isothiocyanate (FITC)-conjugated antiCD4 and electron coupled dye (ECD)-conjugated antiCD28 or corresponding isotypes (both (Beckman Coulter, CA, USA) for 30 min). Cells were washed and 2×10^5 cells were analyzed for their content of CD4+CD28null cells on a Coulter flow cytometer (FC500, Coulter, CA, USA). Percentages of CD4+CD28null cells refer to the total CD4+ cell population. The gating strategy for one representative sample is shown in Fig. 2a.

Proliferation experiments

1×10^5 CD4+ cells, purified from lung tissue of four different COPD patients were incubated with irradiated allogeneic peripheral blood mononuclear cells (PBMC) in the presence or absence of human lung elastin peptides (prepared by enzymatic hydrolysis of human lung elastin), solubilized lung elastin (by successive extractions with hot oxalic acid), and human lung collagen type I (all Elastin Products Company, MO, USA; 30 ng/mL).

Table 1. Demographics of study participants are depicted in Table 1. Groups did not differ in terms of age, gender, and smoking habits

	Gender	Age	FEV_1 %	FEV_1 % VC_{max}	TLC	Medication	Smoker/PY
Patient 1	M	52	29	55	131	Th, ACH, BA, INH-C	Yes/25
Patient 2	M	49	28	39	189	Th, ACH, BA, INH-C	Yes/65
Patient 3	F	65	30	52	141	Th, ACH, BA, INH-C, syst-C	Yes/40
Patient 4	F	57	31	48	160	Th, ACH, BA, INH-C	Yes/30
Patient 5	F	58	15	57	107	Th, ACH, BA, INH-C, syst-C	Yes/38
Patient 6	F	62	13	50	171	Th, ACH, BA, INH-C	Yes/37
Patient 7	F	55	28	62	131	Th, ACH, BA, INH-C, syst-C	Yes/35
Patient 8	F	48	14	47	129	Th, ACH, INH-C, syst-C	Yes/30
Patient 9	M	63	15	36	160	ACH, BA, INH-C	Yes/40
Patient 10	M	58	23	34	132	ACH, BA, INH-C	Yes/100
Patient 11	M	59	23	37	130	ACH, BA, INH-C, syst-C	Yes/50
Patient 12	F	54	17	43	148	Th, ACH, BA, INH-C	Yes/90
Patient 13	F	39	36	51	125	Th, ACH, BA, INH-C, syst-C	No
Control 1	M	61	71	83	120	BA	Yes/80
Control 2	M	87	75	87	109	/	Yes/75
Control 3	M	18	Spontaneous pneumothorax—no lung function available			/	No
Control 4	F	79	61	81	111	/	No
Control 5	M	48	95	93	115	/	No

Th theophylline, *ACH* anticholinergic drugs, *BA* beta-2-adrenergic agonist, *INH-C* inhalative cortisone, *syst-C* systemic cortisone, *FEV* forced expiratory volume, *TLC* total lung capacity

The addition of IL-2 (BD, NJ, USA; 0.6 U/mL) to the experimental setting served as positive control. Plates were incubated for 5 days and then pulsed for 18 h with ^{3}[H]-thymidine. Proliferation was measured in a liquid scintillation counter.

Statistical analysis

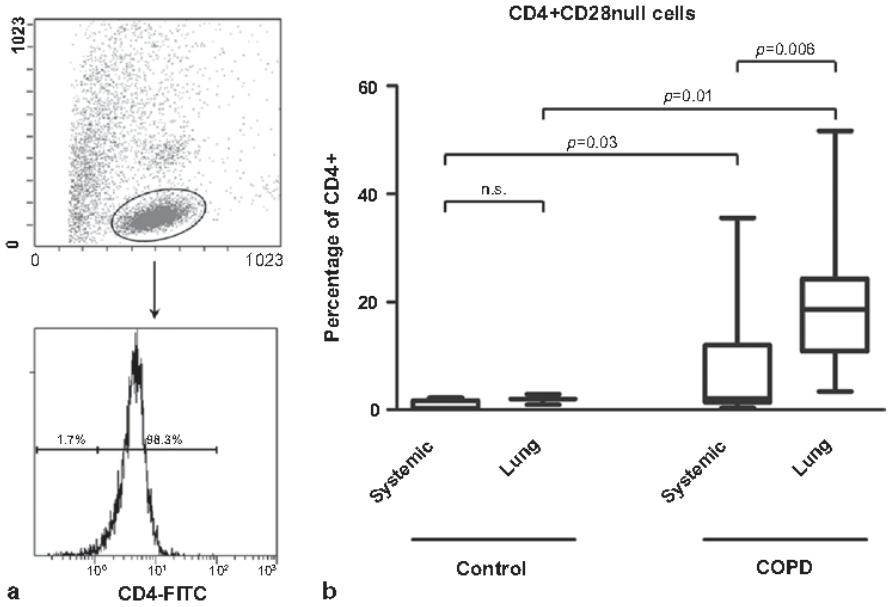
Results are depicted as means ± standard error of the mean and levels of significance were determined by Mann–Whitney test. Data analysis was performed with SPSS 18.0 (SPSS inc., United States) and GraphPad Prism 5 (GraphPad Software Inc., California, USA). A p-value less than 0.05 was regarded as statistically significant.

Results

Patients' characteristics are depicted in Table 1. COPD patients and healthy controls were well matched in terms of gender, age, and smoking habits. Participants from the COPD group were all in Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage IV suffering from a severe airway obstruction. Controls had no evidence of a COPD, according to their lung function.

Increased numbers of circulating CD4+CD28null cells were present in all COPD patients with a mean ± SEM of 8.02 ± 2.9 %. Healthy age-matched controls had only marginal numbers of CD4+ cells lacking CD28 in their circulation (0.79 ± 0.4 %; Fig. 1b). This finding is consistent with previous data published by Lambers et al. [9]. Interestingly, every single COPD patient had a considerably higher number of CD4+CD28null cells in the lung tissue than in the circulation (Fig. 1b and 2a, 20.15 ± 3.9 %; $p = 0.006$).One data set of a representative COPD patient is presented in Fig. 2b. In contrast to that, tissue samples obtained from nonCOPD patients contained almost no CD4+CD28null cells (1.92 ± 0.6 %).

CD4+ cells are thought to be involved in the self-perpetuation of the chronic immune response during COPD. However, conflicting data exist regarding T cell antigens and most work is concentrated on B cell mediated immunity [14–16]. We therefore analyzed prolifera-



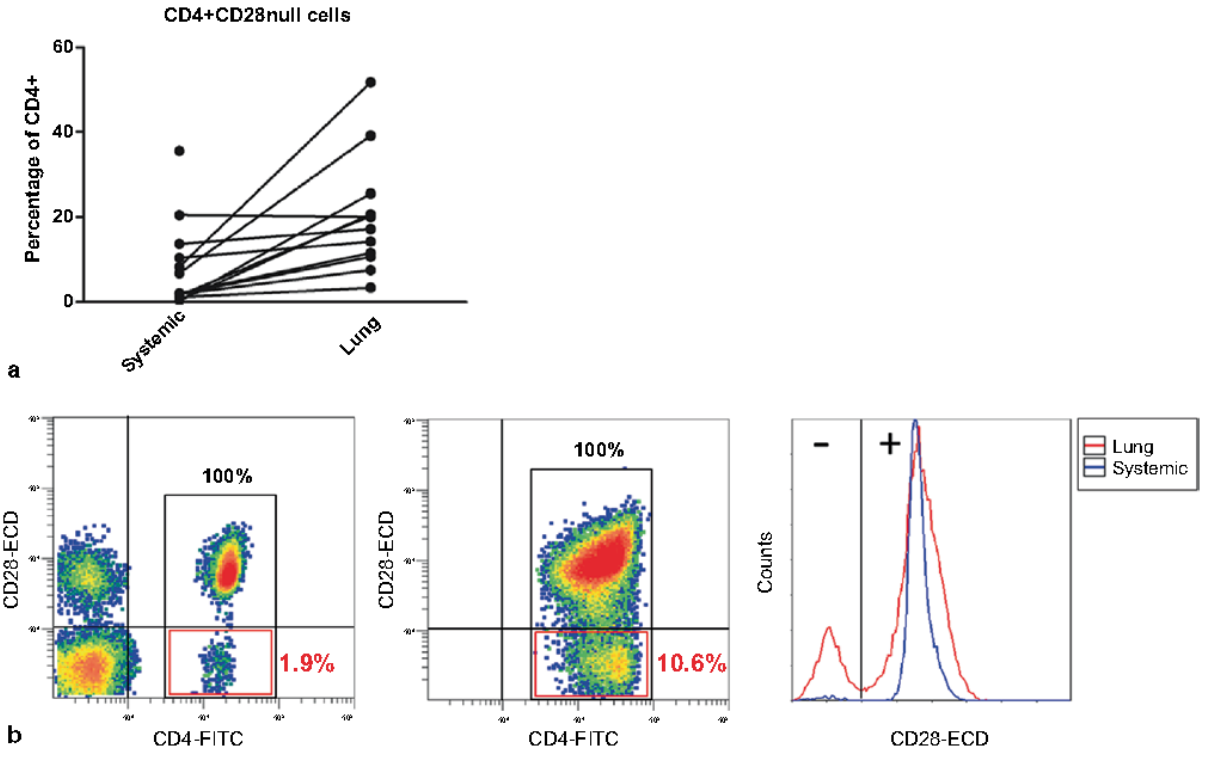


Fig. 1 The purity of CD4+ separation from lung tissue homogenates is depicted in (**a**). Percentages of CD4+CD28null cells of all CD4+ cells in whole blood samples and lung homogenates are shown in (**b**). A marked increment of C4+CD28null cells was seen in the COPD group with highest numbers in the lung resident CD4+ cell population (control systemic: 0.79±0.4 %; COPD systemic: 8.02±2.9 %; control lung: 1.92±0.6 %; COPD lung: 20.15±3.9 %)

Fig. 2 Each COPD patient evidenced an increase of CD4+CD28null in the lung tissue homogenates when compared with systemic levels (**a**; $p=0.006$). A representative case is shown in (**b**)

tive responses of CD4+ T cells isolated from lung tissue of four COPD patients. Coincubation of CD4+ T cells with human lung collagen type I, human lung elastin peptides, and solubilized elastin led to a stable proliferation of lung resident CD4+ T cells (Fig. 3; $n=4$). However, this observation did not lead to levels of statistical significance due to the relatively small sample size.

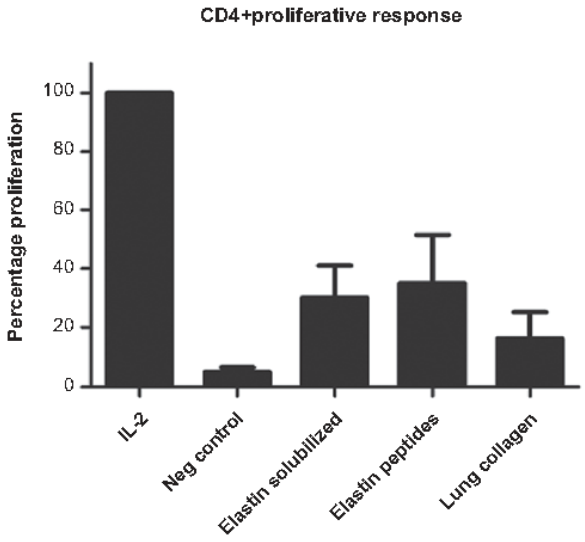


Fig. 3 Proliferative response of lung resident CD4+ cells is shown in Fig. 3. CD4+ cells evidenced a positive response to elastin, elastin peptides, and collagen ($n = 4$)

Discussion

In this study, we showed for the first time that CD4+CD28null cells can be found in high numbers in lung tissue obtained from end-stage COPD patients. Furthermore, lung resident CD4+ T cells showed a proliferative response upon triggering with components of the extracellular matrix.

CD4+ cells, as part of the adaptive immune response, have been shown to be involved in the pathogenesis of COPD. Hogg et al. [6] have thoroughly worked up lung tissue specimen of COPD patients histologically and found a stage-dependent increase of peribronchial CD4+ cells. These results were confirmed by others shortly thereafter [17, 18]. Although an accumulation of CD4+ cells in the small airways of COPD patients is well established, no data on the role of lung resident CD4+CD28null cells exist.

The loss of the costimulatory CD28 molecule is a unique feature of replicatively stressed CD4+ T cells [12]. CD4+CD28null cells have previously been shown to be systemically heightened in several autoimmune diseases including rheumatoid arthritis, Wegener's granulomatosis, multiple sclerosis, and ankylosing spondylitis [10]. These cells are clonally expanded and include autoreactive T cells, implicating a direct role in the development and maintenance of the autoimmune response. We have previously evidenced that CD4+CD28null cells are systemically heightened in COPD patients. Based on flow cytometric analyses, a significant, disease stage-dependent increment of CD4+CD28null cells in blood samples obtained from COPD patients was shown [9]. However, this finding was only partially confirmed by Hodge et al. [13]. Their measurements of CD4+CD28null cells showed a trend towards increased counts in COPD patients, however, did not reach significance. This may be due to the fact that most of the patients included in the study were in mild to moderate GOLD stage. Furthermore, the authors did not provide a stage-dependent analysis.

To date, direct evidence of an involvement of CD4+CD8null cells in the pathogenesis of the chronic tissue destruction in COPD is lacking. We therefore analyzed lung homogenates obtained from end-stage COPD lungs for the presence of CD4+CD28null cells.

CD4+CD28null cell counts were significantly higher in the lung homogenates than in the circulation of COPD patients and were completely absent in healthy controls. Therefore, clonally expanded lung resident CD4+CD28null cells are most likely directly involved in the pathogenesis of COPD. CD4+CD28null cells release high amounts of IFN-γ, a potent stimulator of macrophages, leading to secretion of matrix metalloproteinases and tissue destruction [19]. In addition, CD4+CD28null cells may directly contribute to loss of lung parenchyma by inducing apoptosis of respiratory epithelium [11].

In a further set of experiments of this study, we tried to identify autoantigens contributing to the clonal expansion of lung resident CD4+ T cells. We stimulated purified CD4+ T cells with lung specific components of the extracellular matrix namely, collagen and elastin. Interestingly, both peptides led to a low but stable proliferative response. The importance of this finding is based on the fact that lung resident T cells primarily purified from tissue homogenates were utilized without any steps of enrichment. To the best of our knowledge, this is the first time lung resident CD4+ T cells could be purified in high enough concentrations from COPD patients in order to determine their proliferative capacity.

When autoimmunity came into focus of the COPD research, a response against components of the extracellular matrix was assumed to be a possible key factor. Lee et al. [8] gave evidence of the presence of antielastin antibodies in peripheral blood of patients with severe COPD. Furthermore, this group was able to show a small but stable immune response of peripheral T cells to elastin peptides. Contrarily, immunity against collagen type I, the other major source of the pulmonary extracellular matrix, has not yet been detected. This may be due to the fact that no experiments have been performed with lung resident cells to date.

This work supports the recent autoimmune hypothesis of the pathogenesis of COPD. Future studies should define the clonality of CD4+CD28null cells in COPD and perform a systematic epitope mapping of autoantigens responsible for the detrimental tissue destruction in COPD.

Acknowledgements
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Conflict of Interest
All authors declare no conflict of interest.

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12. Curriculum Vitae

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Family Status: Single

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EDUCATION

2008/10–Present:	Student Research Fellow at the Department of Thoracic Surgery, General Hospital Vienna, Medical University of Vienna, Austria
2006/10 – Present:	Medical Student at the Medical University of Vienna, Austria
2005 – 2006	voluntary Service at the Johanniter-Unfall-Hilfe Austria
2006	graduated at the Bundesrealgymnasium Amerlingstraße
1992-1996	Primary School

CONTINUING EDUCATION

2012/05	Methods Seminar: “Molecular biology”, Univ.-Prof. Mag. Dr. Ellmeier, W, Medical University Vienna
2012/02	Biometry I: Description and Visualization of Medical Data, Medical University Vienna
2012/03	Methods in surgical-immunological research, Dr. Hötzenecker K, Medical University Vienna
2010-2014	Current Topics in Applied Immunology, Medical University Vienna
2010-2014	Applied Immunology and Tissue Regeneration, Medical University Vienna
2013/11	Biometrie II: Statistical Tests and Analysis of Survival in Medical Research, Medical University Vienna
2014/02	Medical English: Lectures and Presentations, Medical University Vienna

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CLINICAL TRAINING

2009/7	Clinical Clerkship at the Emergency Department, Willhelminenspital, Vienna
2009/6	Clinical Clerkship at the Department of Thoracic Surgery, Otto-Wagner-Spital, Vienna
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CONGRESSES AND MEETINGS

2009/09	40. Jahrestagung der österreichischen Gesellschaft für Innere Medizin, Wien
2009/06	50. Österreichischer Chirurgenkongress, Wien
2009/02	53rd Annual Meeting of the Society of Thrombosis and Haemostasis Research, Vienna
2010/06	42. Jahrestagung der österreichischen kardiologischen Gesellschaft, Salzburg
2010/09	41. Jahrestagung der österreichischen Gesellschaft für Innere Medizin
2010/10	24. Jahrestagung der österreichischen Gesellschaft für Transplantation, Villach
2010/12	2nd EACTS Meeting on Cardiac & Pulmonary Regeneration, Vienna
2010/05	52. Österreichischer Chirurgenkongress, Vienna
2011/10	25. Jahrestagung der österreichischen Gesellschaft für Transplantation, Graz
2012/05	Österreichische Kardiologische Gesellschaft Jahrestagung 2012, Salzburg
2012/06	53. Chirurgenkongress, Salzburg
2012/10	Jahrestagung der Österreichischen Gesellschaft für Transplantation, Transfusion und Genetik, Rust
2013/05	54. Chirurgenkongress, Vienna
2014/01	37. Seminar der Österreichischen Gesellschaft für Chirurgische Forschung, Gosau

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ARTICELS

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ETHICS COMITTEE APPROVAL

„Charakterisierung von Psoriasin in Patienten mit Empyem“ **EK: 1605/2013**

METHODS

Cultivation and separation of human and animal cell lines

ELISA

Bradford protein assay

Proliferation Assay

Flow Cytometry

Experience in working with different animal models

SCIENTIFIC VISITS

2010/05 University of Kaposvar, Hungary – Large Animal Experiments

2011/04 University of Kaposvar, Hungary – Large Animal Experiments

2012/02 University of Kaposvar, Hungary – Large Animal Experiments

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COMPUTER SKILLS

MS Word

MS Powerpoint

MS Excel

SPSS

GraphPad Prism

Photoshop

LANGUAGE SKILLS

Native German Speaker

Proficient in English

Knowledge of French