UNIVERSITÀ DEGLI STUDI DI PALERMO

Dottorato di Ricerca in Biomedicina e Neuroscienze - XXV° ciclo

Indirizzo: Medicina Sperimentale e Molecolare

Dipartimento di Medicina Sperimentale

Settore Scientifico Disciplinare BIO/16 – Anatomia Umana

ANTI-INFLAMMATORY ACTIVITY OF HSP60 IN HUMAN BRONCHIAL EPITHELIAL CELLS AND ITS INVOLVEMENT IN CHRONIC OBSTRUCTIVE PULMUNARY DISEASE

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CICLO XXV

ANNO CONSEGUIMENTO TITOLO 2015

1 Introduction

1.1 COPD

1.1.1 Definition of COPD

Chronic obstructive pulmonary disease (COPD) is a disease characterized by chronic airflow limitation that is not fully reversible that causespathological changes of the lung, some with significant extra-pulmonary effectsthat may contribute to the severity in individual patients. The airflow limitation is usually progressive and is associated with an abnormal inflammatory response of the lung to noxious particles or gases, primarily from cigarette smoking.

In the past COPD was known with different names. Bonet described a condition of “voluminous lungs” in 1679. In 1769, Giovanni Morgagni reported 19 cases where the lungs were “turgid” particularly from air. The first description and illustration of the enlarged airspaces in emphysema was provided by Ruysh in 1721. Matthew Baillie illustrated an emphysematous lung in 1789 and described the destructive character of the condition. In 1814 Badham used the word “catarrh” to describe the cough and mucus hypersecretion of chronic bronchitis that was first reported as a disabling disorder. He recognised that chronic bronchitis was a disabling disorder.René Laennec, the physician who invented the stethoscope, used the term “emphysema (1837) to describe lungs that did not collapse as usual because they were full of air and the airways were filled with mucus. In 1842, John Hutchinson invented the spirometer, which allowed the measurement of vital capacity of the lungs. However, his spirometer could only measure volume, not airflow. Tiffeneau in 1947, and Gaensler in 1950 and 1951, described the principles of measuring airflow (Petty TL, 2006).William Briscoe used for the first time in 1965 the term COPD. This term has gradually overtaken other terms to become the name for this disease (Fishman AP, 2005). In 1977 Fletcher and Peto described COPD as an obstructive and hypersecretory chronic disorder of the airways, strictly related to cigarette smoke (Fletcher C., 1977).

Over the years, researchers have done much to help understand the causes, diagnosis, and progression of COPD. Today it is known that a healthy lifestyle (smoking cessation, correct diet, nutrition, and physical exercise) can help people with COPD to manage and improve their symptoms.

1.1.2 Airflow limitation in the airway

Airflow limitation in COPD is caused by presence of an abnormal inflammatory cellular infiltrate in the small airways, remodeling and thickening of the airway wall. The destruction of alveoli and enlargement of airspaces, which are anatomical hallmarks of emphysema, contribute to the loss of elastic recoil and the loss of outward traction of the small airways, leading to their collapse on expiration. This results in airflow obstruction, air trapping and hyperinflation(Hogg JC, 2004). In general, the inflammatory and structural changes in the airways increase with disease severity and persist even after smoking cessation (Tzortzaki EG, 2009).

This characteristic chronic airflow limitation of COPD is caused in part by alterations of the small airways (*chronic bronchitis*) and in part by the destruction of lung parenchyma (*emphysema*); the contribution of each of these two phenotypes varies from individual to individual (Fig. 1).

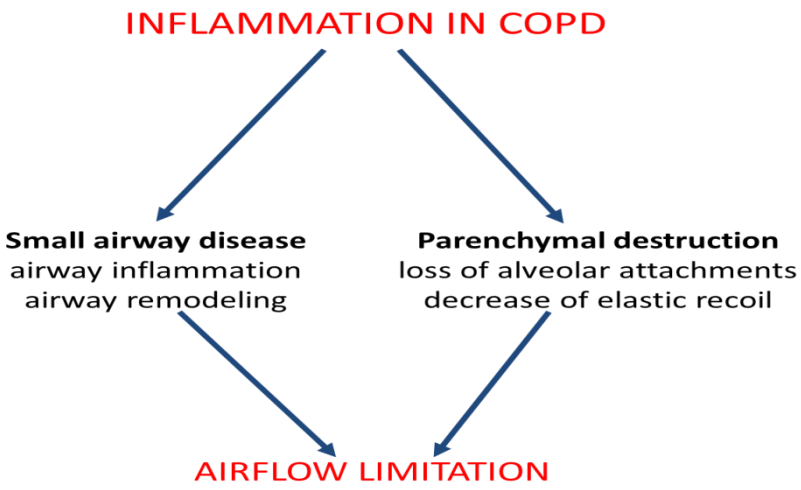


Fig. 1: Mechanisms responsible for airway obstruction in COPD

1.1.3 Chronic Bronchitis

Chronic bronchitis is characterized by cough and sputum production for at least 3 month in each of two consecutive years. The symptoms may precede the development of airflow limitation by many years. These symptoms may occur in the natural history of COPD with varying severity and at different times (Tager IB, 1976). Hypersecretion

of mucus in the proximal airways, responsible for chronic bronchitis, however, does not correlate with the decline in lung function (FEV) in patients with COPD (Peto R, 1983).Chronic inflammation and secretions are the obstructive component of the disease and cause structural changes and the restriction of the small airways. In contrast to emphysema, chronic bronchitis is associated with a relatively undamaged pulmonary capillary bed. Emphysema is present to a variable degree but is usually centrilobular rather than panlobular. The body responds by decreasing ventilation and increasing cardiac output (ventilation/perfusion (V'/Q') mismatch) leading to hypoxaemia, polycythaemia and increased CO_2 retention, and eventually these patients develop signs of right hear failure (Tzortzaki EG, 2009).

1.1.4 Pulmonary Enphysema

The second major COPD phenotype is the emphysematous patient. *Emphysema* is defined by destruction of the lung parenchyma, also by inflammatory processes, causes the gradual loss of alveolar septae in the small airways and a reduction in lung elastic recoil (Penman RW, 1970;Colebatch HJ, 1973). These changes decrease the ability of the airways to remain open during expiration, leading to decreased ability to oxygenate blood. Eventually the rest of the body suffers from tissue hypoxia, pulmonary cachexia, muscle wasting and weight loss (Groneberga DA, Fan Chung K, 2004).

1.1.5 Exacerbations

Exacerbations have a significant impact on morbidity, disease progression, disability and socio-medical costs (Celli BR, 2007). Bacterial or viral infections, often overlapping with other factors such as environmental pollutants are the principal cause of exacerbation. From a clinical point of view, exacerbations may lead to an increased cough, change in sputum characteristics, that becomes more abundant and/or purulent, appearance of wheezing/whistling expiratory and worsening of dyspnea. During the course of the disease, the frequency and severity of exacerbations tends to increase, resulting in accelerated decline in lung function (Brill SE, 2014).

1.1.6 Comorbidity and Systemic Involvement

Comorbidities contribute to the overall severity in individual patients (Global Initiative for Chronic Obstructive Lung Disease, Revision 2013). Moreover, COPD also produces significant systemic consequences mainly due to the development of the systemic inflammation.

The characteristic symptoms of COPD are dyspnea (difficulty breathing and "air hunger"), cough and sputum overproduction: cough and sputum production may precede many years the development of airflow limitation. As COPD tends to develop in people who smoke for a long time (Schirnhofer L. et al., 2007), patients often have comorbidities related to smoking or aging. COPD itself also has significant extrapulmonary systemic effects that lead to comorbid conditions (Barnes PJ et al., 2009) significantly affecting the quality of life and survival of affected individuals. Weight loss, nutritional abnormalities and skeletal muscle dysfunction are well-recognized as secondary effects of COPD and patients have an increased risk of myocardial infarction, osteoporosis, bone fractures, depression, diabetes, sleep disorders, anemia and glaucoma (Van Weel C., 1996). A differential diagnosis and comprehensive assessment of severity of comorbid conditions should be conducted in each patient with a reduction in chronic airflow (fig. 2).

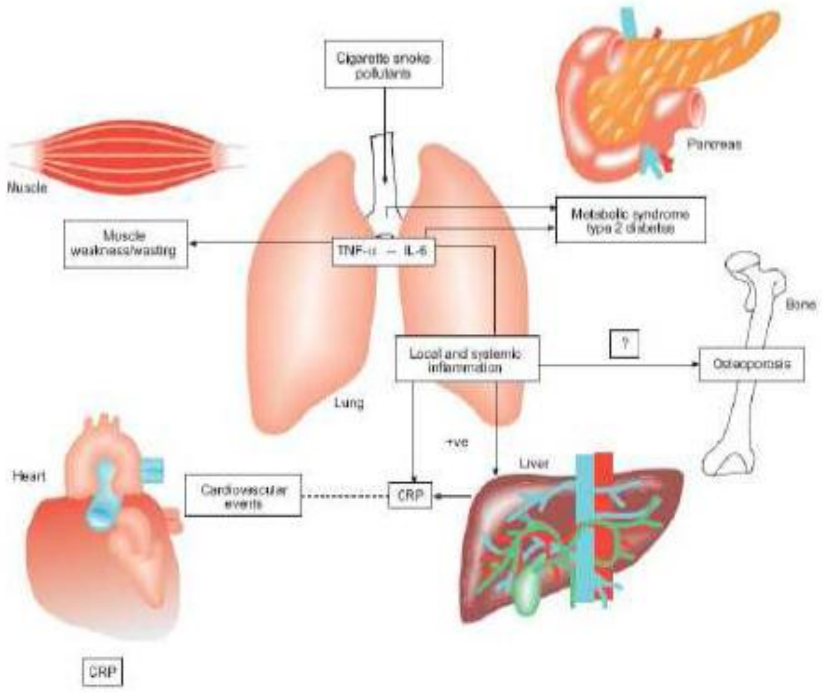


Fig 2:The central role of inflammation in comorbidity is associated with COPD. Inflammation appears to play a central role in the pathogenesis of COPD and other conditions that are increasingly being recognized assystemic inflammatory diseases. As part of the chronic inflammatory process, tumour necrosis factor (TNF)-a receptor polymorphisms are associated with increased severity of disease, possibly due to enhanced TNF-a effects. Also, C-reactive protein (CRP) levels can be increased directly by TNF-a and other cytokines. Elevated CRP and fibrinogen may be crucial in the pathogenesis of cardiovascular disease. Reactive oxygen species released as a result of COPD may enhance the likelihood of a patient developing cardiovascular disease, diabetes and osteoporosis.. From Fabbri LM et al, EurRespir J 2008.

1.1.7 Natural History

COPD has a variable natural history and not all individuals follow the same course. However, it is usually a progressive disease, especially if the patient's exposure to noxious agents continues. Once triggered, COPD and its comorbidities become chronic leading to continuous therapy

1.1.8 Diagnosis

The best way to measure the level of airflow limitation is spirometry, which is the most common and reproducible pulmonary function tests. This examination is required after patient analysis if there are evidences that lead to suspect a possible chronic obstructive bronchitis. The clinical diagnosis of COPD should be considered in all patients present with dyspnea, chronic cough or sputum production and a history of exposure to risk factors for the disease (Zwar NA et al., 2011). Spirometry is essential for diagnosis and provides a useful measure of the severity of pathological changes: measures the volume of forced exhaled air from maximal inspiration (forced vital capacity, FVC) and the volume of air exhaled during the first second of this maneuver (forced expiratory volume in one second, FEV1). Then is calculated the ratio of these two measurements (FEV / FVC).The spirometry should be performed after adequate dose of bronchodilator with short duration of action, that reduce the variability of the test. A value of FEV1/FVC < 0.70 after bronchodilator allows confirmation of persistent airway obstruction(Fig 3).However, since the aging affect lung volumes, the use of a fixed value can lead to an overestimation of the diagnosis of COPD in older people.

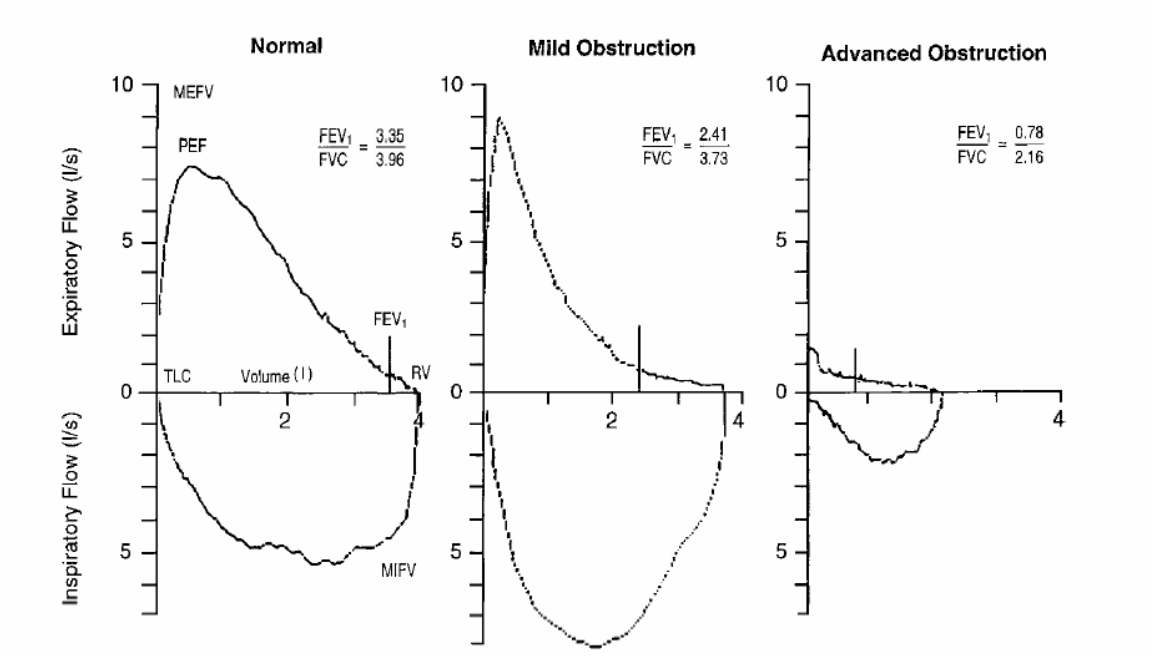


Fig. 3: Graph of the curve spirometric flow/volume. In advanced stages of COPD, there is a marked reduction in FEV1, FVC and maximal expiratory flow in general. The curve flow/volume in the expiratory phase flattens out. Even the inspiratory flow undergoes a reduction but less pronounced than that expiratory (McGraw-Hill 2008)

1.1.9 Evaluation of Gravity Disease

In previous documents the severity of COPD was defined only on the basis of the degree of airway obstruction, assessed by spirometry in terms of value compared with the theoretical FEV_1(fig. 4):

- GOLD 1: Mild, FEV> 80% predicted
- GOLD 2: Moderate, 50% <FEV <80% predicted
- GOLD 3: Severe, 30% <FEV <50% predicted
- GOLD 4: Very Severe, FEV <30% predicted FEV.

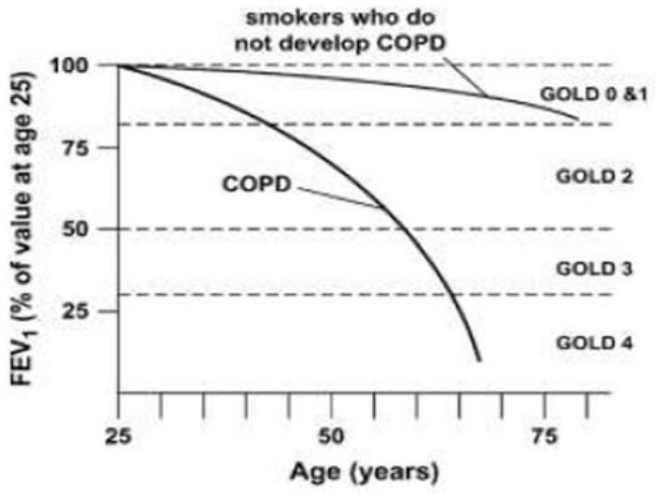


Fig 4. The natural history of the FEV1 decline in men followed by Fletcher et. al is shown with theGOLD severity stage superimposed as dotted horizontal lines. Modified from Curtis JL et al., Proc Am ThoracSoc 2007

The new GOLD guidelines (Revision 2013) underlines the diagnostic, therapeutic and prognostic importance of a more comprehensive assessment of disease stage and severity, through the integration of anamnestic and clinical data with spirometry. These include the analysis of the patient's symptoms, the evaluation of spirometric measurements and the risk of exacerbations. Regarding the symptoms, are used questionnaires: the mMRC (British modified Medical Research Council), the CAT (COPD Assessment Test) and the CCQ (Clinical COPD questionnaire). The first allows the evaluation of the degree of patient's breathlessness: must be ranged from grade 0, in which the patient claims to have dyspnea only in case of intense efforts, to grade 4, in which the patient complains shortness of breath even at rest (Bestall JC et al., 1999). The CAT assess the state of deterioration of health status through 8 questions (http://www.catestonline.org) and provides a score from 0 to 40. The CCQ is a questionnaire for measuring clinical control in patients and identify cases you need to change the treatment plan (Van Der Molen T et al., 2003).

Another key tool for staging and determining the severity of COPD results to be even spirometry. In patients who have obtained, spirometric analysis, a ratio FEV1/FVC less than 0.7 measurements are evaluated by comparison with reference values in relation to age, height, sex, and race (Johannessen et al., 2006). The severity of the disease is stable as a percentage of FEV1 compared to the reference value.

Stage I - Mild COPD: mild airflow limitation (FEV_1/FVC <0.70; FEV ≥ 80% predicted). Chronic cough and sputum production may be present or not. The patient often does not recognize the reduced lung function.

Stage II - Moderate COPD: worsening of airflow limitation (FEV_1/ FVC <0.70; 50% ≤ FEV <80% predicted), with shortness of breath typically after effort and cough with sometimes present expectoration. The patient usually requires medical attention.

Stage III - Severe COPD: further worsening of airflow limitation (FEV_1/FVC<0.70; 30% ≤ FEV <50% predicted), greater shortness of breath, reduced exercise capacity, and easy fatigability.

Stage IV –Very Severe COPD: severe airflow limitation (FEV_1/FVC<0.70; FEV <30% predicted) with chronic respiratory insufficiency. This may also have consequences on the heart. In this stage, quality of life is considerably compromised and it is at risk life itself.

Another parameter used to determine more accurately the stage of COPD patients is the risk of exacerbations: a COPD exacerbation is defined as an acute event characterized by worsening of respiratory symptoms of the patient that is beyond normal day-to-day variations and leads to a change in treatment plan: the number of exacerbations varies considerably from patient to patient (Burge S.et al., 2003). It analyzes the patient's history and assesses the clinical history of previous exacerbations (two or more per year) to determine the risk of future exacerbations.

Until few years ago the severity of the disease was determined by analysis of one-dimensional spirometric classification (fig. 4); However, the weak correlation between FEV_1, as determined by spirometry, symptoms, and reduced quality of life (Jones PW, 2009) led to revise a new method for the classification of COPD severity. Today are jointly assessed symptoms, spirometric classification and the risk of exacerbations: Patients are divided into four groups (fig. 5). This combined approach reflects better the complexity of COPD compared to the analysis of one-dimensional airflow limitation and allows a more accurate choice of treatment.

Patients are divided into one of four categories shown in Table 1:

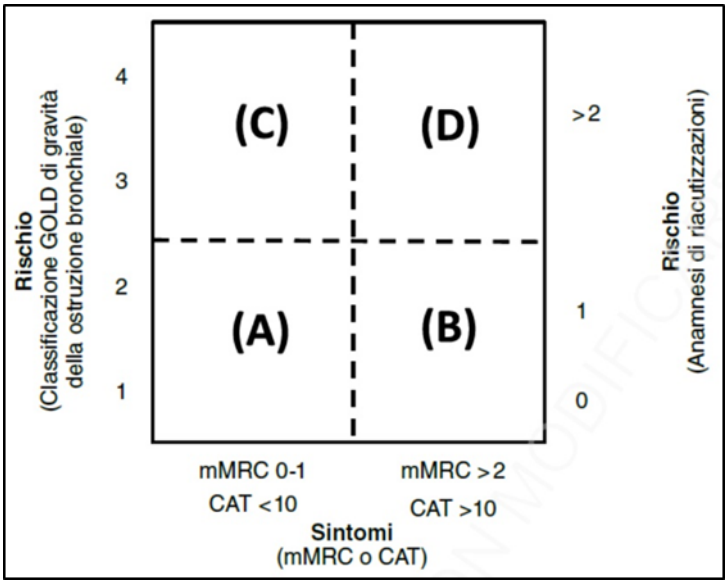


Fig. 5:The combined assessment of COPD: association between symptoms, spirometric classification and future risk of exacerbations.

Table 1: characteristics of the four COPD groups deriving from combined analysis

Patient	Characteristics	Spirometric classification	Exacerbations per year	mMRC	CAT
A	Low risk Mild symptoms	GOLD 1-2	≤ 1	0 - 1	< 10
B	Low risk severe symptoms	GOLD 1-2	≤ 1	≥ 2	≥ 10
C	High risk mild Symptoms	GOLD 3-4	≥ 2	0 - 1	< 10
D	High risk Severe symptoms	GOLD 3-4	≥ 2	≥ 2	≥ 10

1.1.10Epidemiology

COPD is one of the major cause of morbidity and mortality worldwide.In 1990 it was the sixth leading cause of death. The growing burden of COPD is partly due to the ageing of the world's population and partly to the continued use of tobacco(Lopez AD, 2006). Thisprojected COPD to be the fourth leading cause of death by 2020,resulting a significant economic and social costs, like in the U.S. where COPD in 2007 cost $42.6 billion in health care costs and lost productivity.(Global initiative for Chronic Obstructive Lung Disease, 2013). In 2030it is estimated to become the third leading cause of death (World Health Organisation, 2008.)

Cigarette smoking is the main risk factor for COPD worldwide (Ballwed K., 2014), although, in many countries, even the air pollution that comes from solid fuels (wood, animal dung, agricultural wastes,charcoal and coal) has been identified as a possible risk factor (Pope D. et al., 2014).

The epidemiology varies considerably between countries and between groups of people members of the same nation. This difference may be due to different source of exposure and individual susceptibility to risk factors and to different methods and criteria for diagnosis. The Burden of Obstructive Lung Disease Study (BOLD) (Buist AS, 2007), conducted in 12 different locations of the world on 9425 patients, reported the following data:

- Prevalence of disease progressively increase with age in both males and females.
- Higher prevalence in males.
- Increased prevalence in parallel with the increase in the number of pack-years (number of cigarettes per day multiplied by years of smoking divided by 20).
- Substantial prevalence of COPD even in non-smokers: 11.3%.

Most of the available epidemiological data collected suggest that the diagnosis of COPD is found in 4–10%of the population,but it is reasonable to assume that the disease still remains under-diagnosed. A lot of data come from Europe and North Americacountriesand it is not possible to locate any spirometric studies reporting COPD in the African or Eastern Mediterranean regions. In addition, only few reports were found from the South-East Asian and Western Pacific regions (Halbert RJ, 2006). Chan-Yeung et al. report a statistical model to estimate the prevalence of moderate and severe COPD in the Asia–Pacific region, with a regional estimate of 6.3% and projected country-specific rates of 3.5– 6.7% (Chan-Yeung M, 2004).

Estimate the diffusion of COPD worldwide is therefore very difficult, considering that in many developing countrieslacks the ability to perform spirometry, essential for the diagnosis.

The slow evolution of the natural history of the disease and the lack of specific symptoms, especially in the early stages, are the main cause of the delay with which patients come to medical attention. Moreover, the absence of valid screening programs poses great risk to the population (Van de Boom G, 1998).

Despite these complexities, emerging data are not valid to draw conclusions about the prevalence of COPD. A systematic review and meta-analysis studies conducted in 28 countries between 1990 and 2004 (Fukuchi Y et al., 2004; Halbert RJ et al., 2006) provide evidence of the prevalence of COPD significantly higher in smokers and ex-smokers than non-smokers, in subjects over 40 years and higher in men than in women.

1.1.11Mortality

Although COPD is often a primary cause of death, it is considerederroneously as a contributory cause.It is frequently totally omitted from the death certificate and the death is attributed to another condition, such as cardiovascular disease. The underestimation of many cases of COPD influence much on mortality data, but nevertheless the problems with the accuracy of mortality data, COPD is considered one of the important causes of death in most countries. Currently represents one of the major causes of death worldwide and according to a recent study, by 2030 will become one of the first leading cause death (Mathers et al., 2006), mainly as a result of the increase of smoking and increased life average(fig. 6).

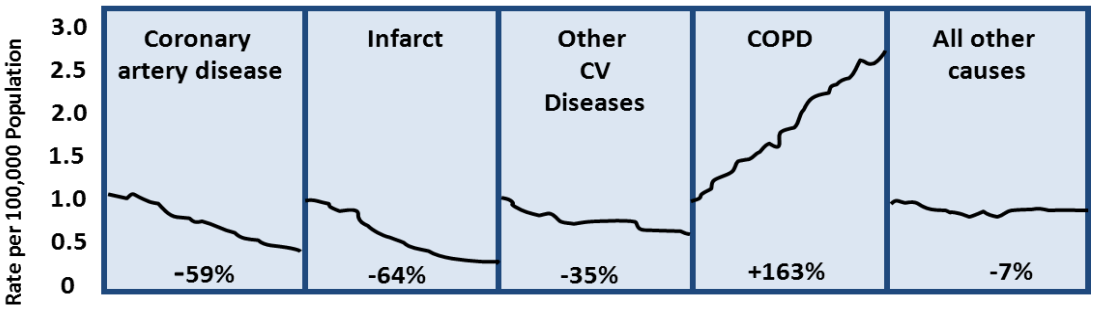


Fig. 6: Percentage variation of mortality in the U.S.A. (age-adjusted) from 1965 to 1998

1.1.12Risk Factors

The etiology of the disease is not completely clear, but it is know that COPD is a multi-factorial disease. Many studies have been conducted analyzing the diseasecourse in a lot of patients: none of these, however, analyzed the early onset of the disease, determining to understand the cause. The COPDrisk factors are therefore not yet

entirely understand; the certainty seems to be that this disease may be triggered by gene-environmentinteractions.

Identification of cigarette smoke as a major risk factor led to consider smokingcessation programs an essential element in the COPDprevention. Although smoking is the most studiedrisk factor, several studies indicate that even non-smokers may develop chronic airway obstruction (Lamprecht B et al., 2011)(fig 7).

Risk factors currently identified (new2.goldcopd.it, 2014) are:

- Genetic Factors
- Exposure to pollutants
  - Tobacco Smoke
  - Organic and Inorganic Professional Powders
  - Domestic pollution caused by cooking food and heating with biomass in poorly ventilated environment
  - Air pollution
- Altered growth and lung development
- Sex
- Age
- Respiratory infections
- Socio-economic Status
- Chronic Bronchitis
- Asthma

1.1.12.1 Genetic Factors

COPD is a polygenic disease and it constitutes an example of gene-environment interaction. A significant familial risk of airflow obstruction was observed in smoking siblings with severe COPD, suggesting an important role of genetic factors in the susceptibility of thedisease(McCloskey et al., 2001).

The best documented genetic risk factor is a severe hereditary deficiency of alpha-1 antitrypsin (Stoller JK et al., 2012). This is an inhibitor of the serin-protease that in the lung prevents tissue damage by proteases (such as elastase released from neutrophils). A deficit (or loss of function) of alpha-1 antitrypsin exposes the lungs to

possible irreversible damage. This condition is more common in Northern European subjects(Blanco et al., 2006).

The prematureand accelerated development ofpanlobular emphysema and the decline in lung function appear in both smokers and nonsmokers with severe deficiency, although smoking increases the risk. There is a great variability between individuals, in the extent and severity of emphysema and decline in lung function. Through linkage studies have been identified several regions of the genome containing presumably susceptibility genes for COPD: the genes encoding the metallo-proteinase 12 (MMP12) (Hunninghake GM. et al., 2009), the transforming growth factor beta 1 (TGFβ- 1) (Wu L.et al., 2004), the hedgehog interacting protein (HHIP) and the nicotinic acetylcholine receptor alpha (αnAchR) (Pillai SG et al., 2009) appear to be involved in the onset of COPD. On the other hand, these association studies have many limitations and allelic variants that influence the development of COPD (other than alpha-1 antitrypsin deficiency) have not been definitively identified (Silverman EKet al., 2002).

1.1.12.2 Exposure to pollutants

All individuals in the course of their lives are exposed to various types of inhalable particles. It is therefore useful to think in terms of the total amount of inhaled particles. About the many inhalational exposures that may be encountered, only tobacco smoke (Tashkin DP, 2009) and exposure to dusts and chemicals (vapors, irritants, and fumes) in a workplace (Matheson MC et al., 2005) recognized as agents capable of causing COPD.

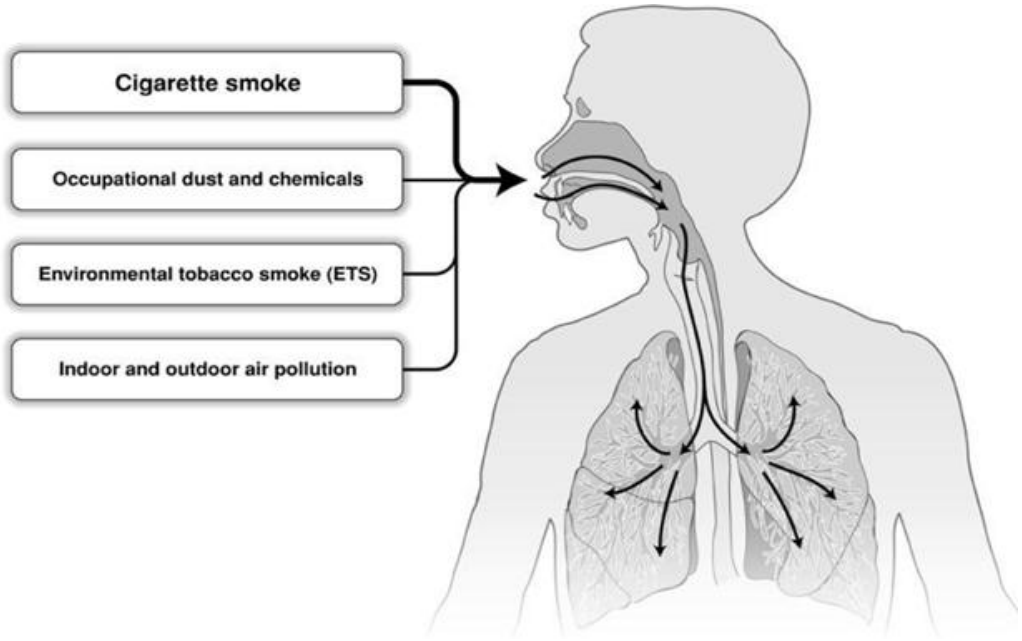


Fig 7. The risk of developing COPD is related to the total burden of inhaled toxic particles

1.1.12.3 Tobacco smoke

Tobacco smoke is the major risk factor for COPD development that is frequentlyencountered. Smokers have a higher prevalence of respiratory symptoms and abnormal lung function, a higher annual rate of FEV_1decline and a higher mortality rate for COPD compared with non-smokers subjects (Kohansal et al., 2009). In addition to cigarettes, other types of tobacco are risk factors, such as pipes, cigars, water pipe (Raad et al., 2011) and marijuana (Tan WCet al., 2009). The age at which you start smoking, the number of cigarettes smoked and the current smoking status are predictors for mortality from COPD. However, not all smokers develop clinically significant COPD, suggesting a role of genetic factors. Even passive smoke plays an important role and it is one of the possible inhaleragents that can predispose to COPD: exposure to secondhand smoke (environmental tobacco smoke, ETS) may contribute to respiratory symptoms (Office on Smoking and Health, 2006) and COPD (Eisner MD et al., 2005), increasing the burden of inhaled particles and gases in the lung. In pregnant woman, smoking may be a risk to the fetus, influencing the growth and development in the uterus (Tager IB et al., 1995).

1.1.12.4 Occupational exposure to Dusts and Chemicals

Exposures to pollutants in the workplace are an underestimated risk factor for COPD. These exposures include organic and inorganic dusts, chemicals and fumes. Individuals exposed to these factors in the workplace have an increased risk of developing bronchitis, emphysema and COPD (Matheson et al., 2005). In developing countries, where workplaces are less controlled, the risk for occupational exposure is probably higher than reported in European and North American studies.

1.1.12.5 Indoor pollution

Wood, animal dung, crop residues and coal, typically burned in open fires, and malfunctioningstoves can lead to high levels of indoor pollution (Oroczo M-Levi et al., 2006). The biomass used for cooking and heating indoor environments are proving to be an important risk factor for COPD (Torres-Duque C et al., 2008) (Ezzati M., 2005).

1.1.12.6 Air Pollution

High levels of urban air pollution are harmful to individuals with existing heart or respiratory disease. It is difficult to draw a correlation between air pollution and COPD, but it seems to be a risk factor less influent than the cigarette smoke. However, there is a correlation between urban air pollution, mainly due to emissions of exhaust gases from motor vehicles, and reduced lung function (Abbey DE et al., 1998). The role of the inhalation of airborne contaminants is a risk factor not completely understoodtoday.

1.1.12.7 Other risk factors

In addition to the factors described above, counted among the greatest risk factors for the onset of the disease, there are other factors for which has been found a correlation with COPD.

1.1.12.8 Lung growth and development

Lung growth is correlated to processes occurring during pregnancy, birth, and exposures during infancy and adolescence (Barker DJ, 1991). Any factor that acts on the development of the lungs during pregnancy and childhood, such as birth weight (Lawlor DA, 2005) or respiratory infections in early age (De Marco R et al., 2011), has the ability to increase an individual's COPDrisk (Stern DAet al., 2007).

1.1.12.9 Gender

The role of gender as a risk factor of COPD is unclear. In the past, many studies showed a higher prevalence of COPD and mortality in male than female. Recent studies contradict what is shown in the past by identifying, in both sexes, about the same prevalence and mortality of COPD (National Heart, Lung, and Blood Institute, 2009), which probably reflects changes in habits to tobacco smoke (Silverman EK, 2000). Some studies also suggest that women are more susceptible to the harmful effects of smoking than men (Sørheim IC et al., 2010).

Age is often referred to be a risk factor for the development of COPD, although it is unclear whether an aging health conditions lead to COPD or if age reflects instead the amount of cumulative exposures during life.

1.1.12.10 Infections

Infections (viral or bacterial) can contribute to the pathogenesis and progression of COPD. A history of severe infancy respiratory infection has been associated with a reduction in lung function and an increase in respiratory symptoms in adults (Svanes et al., 2010; De Marco R et al., 2011). Susceptibility to infection is correlated with birth weight, considered another risk factor for COPD.

Infections play a very important role in the frequent exacerbations hitting affected individuals (Sethi S, 2010; Wark PA et al., 2013), while the effect on the development of the disease is less clear. Some studies show that diseases with bacterial etiology such as tuberculosis, can play a role in the development of COPD (Menezes AM et al., 2007), although it is difficult to understand the cause-effect relationship between the two diseases. Recent studies have shown that HIV infection accelerates the

onset of pulmonary emphysema due to smoking (Crothers K HL et al., 2011). HIV contributes to exacerbate the inflammatory situation and the immunosuppression consequent to infection can promote the development of pathogenic bacterial flora in the lung.

1.1.12.11 Socioeconomic Status

There is an evidence that the risk of developing COPD is inversely correlated to socioeconomic status (Prescott et al., 1999) even there are unclear components involved in determining this susceptibility: could be a reflection of pollution of indoor and outdoor environmentsas well as crowding, poor nutrition, or the increased risk of infection.

1.1.12.12 Asthma

Do not exist conclusive evidence, but it is assumed that asthma associated with bronchial hyperreactivity may be a risk factor for the development of COPD (Silva GEet al., 2004).

1.2 Pathology, Pathogenesis and Pathophysiology

The inhaled cigarette smoke and other noxious particles cause lung inflammation, a normal response which appears to be amplified in patients who develop COPD. This abnormal inflammatory response may induce alterations in the bronchial and parenchymal tissue destruction (resulting in emphysema) and may alter the normal defense and repair mechanisms(leading to fibrosis of the small airways lung). The pathological changes lead to air trapping and progressive airflow limitation.

1.2.1 Anatomo-Pathology

The anatomo-pathology characteristic of COPD include chronic inflammation, with increased numbers of specific inflammatory cells in several parts of the lung, and structural changes resulting from repeated damage and attempted repair. These anomalies are detectable in the proximal and peripheralairways, in parenchyma and pulmonary vessels (Hogg JC, 2004). In the proximal airways (trachea and bronchi with diameter> 2 mm) there is an increase of goblet cells, enlargement of submucosal glands (both due to hyper-secretion of mucus) and squamous metaplasia of the epithelium.

In peripheral airways (bronchioles <2 mm internal diameter) can be found wall thickening, peribronchial fibrosis, inflammatory exudate and lumen narrowing (obstructive bronchiolitis): the exudateandthe inflammatory response increase with the worsening of the disease. The lung parenchyma (respiratory bronchioles and alveoli) has a damagedalveolar wall with epithelial and endothelial apoptotic cells. Emphysema is characterized by dilatation and destruction of the bronchioles. Inpulmonary vessels the intima is thickened and there is an increase of smooth muscle, due to pulmonary hypertension.

1.2.2 Pathogenesis

The inflammation of the respiratory tract in patients with COPD is revealed as an amplification of the normal inflammatory response to chronic irritants such as cigarette smoke. The mechanisms underlying this amplifiedinflammation are still not completely clear: the persistent presence of an inflammatory situation is associated with

chronic cough andexacerbatedsputum hyperproduction. The hypothesis to explain the chronic inflammation involves the activation of macrophages and epithelial cells in response to harmful stimuli, which ensure the production of molecules with inflammatory activity and cytokines with chemotactic activity against mainly neutrophils. The release of proteases is the main cause of anatomo-pathology alteration in COPD (Barnes, 2004) (fig. 8).

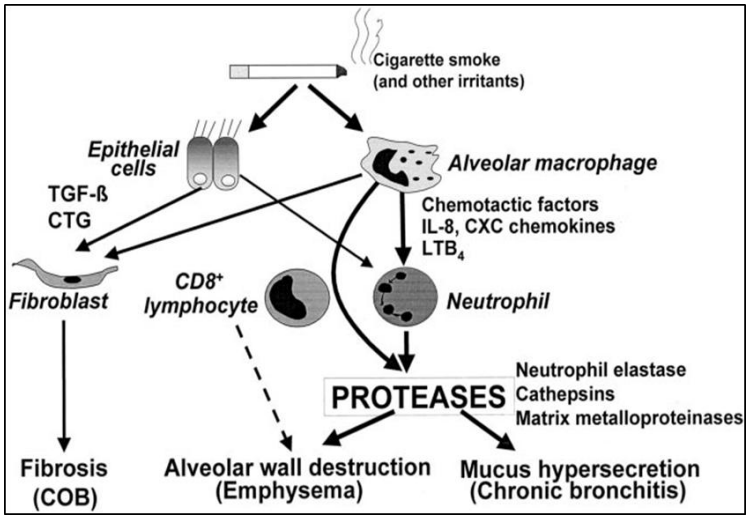


Fig. 8: Inflammatory mechanisms in COPD: smoke and other irritants activate alveolar macrophages and epithelial cells that release chemokines that attract neutrophils with subsequent production of protease harmful to lung tissue

However, some patients develop COPD without smoking: remains unknown the origin of the inflammatory response in these cases (Birring SS et al., 2002). In general, inflammation and structural changes in the airways increase with disease severity and persist after cessation of smoking.

1.2.3 Inflammatory Cells

COPD is characterized by a specific inflammatory pattern involving neutrophils, macrophages and lymphocytes (Barnes PJ et al., 2003; Di Stefano et al., 2004). Neutrophils play a leading role in the COPD pathogenesis. Their number increases in sputumof healthy smokers and further increases in COPD patients according to the severity of the disease. Neutrophils release proteases, inducing the degradation process of the lung parenchyma and playing an important role in the mechanisms of mucus

hypersecretion. Macrophages, derived from circulating monocytes,are considerably increase in airway lumen, lung parenchyma and in bronchoalveolar lavage (BAL). Studies in bronchial biopsies of patients with COPD with increasing severity, showed an increase of neutrophils and macrophages (CD68 +) (Di Stefano et al., 2004). Neutrophils are active in the bronchial mucosa in patients with COPD (Di Stefano et al. 2009). IL-7, IL-27 but not the molecular complex that constitutes the inflammasome are increased in patients with COPD (Di Stefano et al., Thorax 2014).

T cells are increased in the airway wall and lung parenchyma with increased ratio CD8 + / CD4 + in relation to the severity of the disease. The CD8+ T cells may exert their cytotoxic effect on alveolar cells contributing to their destruction. B cells are also found increased in COPD, probably in response to chronic colonization and infection of the airways. Eosinophils not seem to play a primary role in the COPD pathogenesis, although affected individuals show an increase in eosinophil source.

1.2.4 Inflammatory Mediators

All the cells mentioned above, in addition to epithelial cells, release inflammatory mediators that trigger chronic lung inflammation in COPD. The wide variety of inflammatory mediators, which have proven increased in patients with COPD (Barnes et al., 2004), attract inflammatory cells in the respiratory tract (chemotactic factors), amplify the inflammatory process (cytokines) and inducing structural changes (growth factors). Among the chemotactic factors, an important role is played from interleukin 8, found significantly increased in patients with COPD (K Larsson, 2008).

The IL-8 (CXCL8) is a potent chemoattractant of neutrophils: there is a correlation between the concentrations of IL-8 and bacterial counts performed on sputum of COPD patients, suggesting the role of this chemokine in the induction of neutrophilic inflammation in response to bacterial infection of the airways (Patel et al., 2002). This molecule is also increased in the bronchoalveolar lavage supernatants of COPD patients and correlates positively with the number of neutrophils. It is synthesized by many cells, especially epithelial cells and macrophages. Bronchial epithelium secretes IL-8 in response to contact with various agents including LPS (lipopolysaccharide), TNF-α and cigarette smoke. Primary epithelial cells taken from COPD patients grown in culture reveal a production of IL-8 much higher compared to

cells taken from healthy smokers (Schulz et al., 2004). These findings have led to the development of a therapy with monoclonal antibodies directed against IL-8 to try to arrest the inflammation in COPD (Mahler DA et al., 2004).

1.2.5 Respiratory Epithelium

The respiratory epithelium is a pseudostratified, cylindrical, ciliated epithelium with numerous goblet cells (fig. 9).

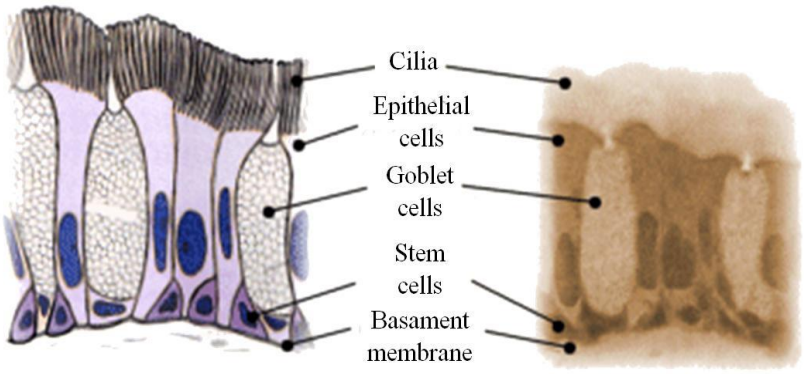


Fig. 9: Pseudostratified epithelium present in the mucosae of the respiratory tract with the coexistence of different cell types: epithelial cells, mucus goblet cells (Goblet cells) and basal stem cells. All cells lean on a basement membrane and the nuclei are arranged at different levels: the cells of the basal layer have an intense mitotic activity and they produce new cells that replace mature cells, lost or damaged.

It forms the first barrier toward inhaled insults, separating lung tissue from the environment. Consequently, epithelial cells are one of the first cells to be exposed to inhaled noxious gases and particles present in cigarette smoke and diesel exhaust fumes. An increase in apoptotic epithelial cells has been shown in the lungs of emphysema patients (Kasahara, Y. et al. 2001; Tuder, R.M. & Petrache, I. 2012; Pouwels SD et al. 2014) (fig. 10) The airway epithelium covers the entire respiratory tract with the exception of the lower portion of the pharynx and alveoli. The goblet cells, together with the mucous glands which are located below the epithelium, produce a thick and viscous mucus that covers the external surfaces of the epithelium. In the nasal cavity, the cilia have the function of directing all foreign particles or micro-organisms that are trapped in the mucus towards the pharynx, from which it can pass through the esophagus into the stomach, where they are neutralized by gastric juice. Even in the

lower portions of the respiratory airway, cilia move in the direction of the pharynx, keeping free the air passage. The airways surface is delicate and can also be severely damaged if are inhaled pathogens and detritus. The presence of irritants along the airways lining can cause abscess formation, while the damage to the respiratory epithelium affected areas may allow irritants to penetrate the lung parenchyma. The scar tissue that is formed reduces the elasticity of the lungs and can might also restrict the airway lumen. Irritants or foreign particles can also penetrate into the pulmonary lymphatic vessels, causing inflammation of the lymph nodes.

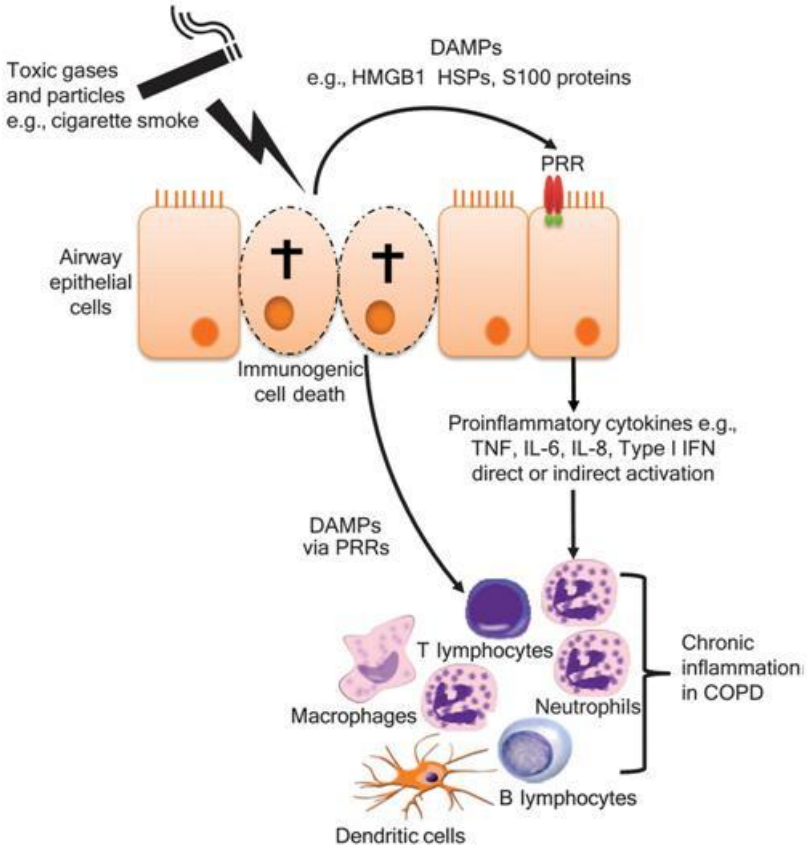


Fig. 10: Hypothetical scheme of cigarette smoke (CS)-induced airway epithelial immunogenic cell death followed by damage-associated molecular pattern (DAMP) release and subsequent triggering of the innate and adaptive immune responses in chronic obstructive pulmonary disease (COPD). Inhalation of toxic gases and particles, e.g., cigarette smoke and diesel exhaust particles, causes damage and subsequent cell death to airway epithelial cells.Modified fromPouwels SD et al. 2014. Mucosal Immunol.7(2):215-26

Chronic inflammation in COPD is due to massive activation of inflammatory cells, especially macrophages, CD8 T lymphocytes and neutrophils. In recent years, many studies are concentrating on the possible role of epithelial cells, eliminating the previous convictions who saw it as an innocent bystander, involved in more physical

defense than in inflammatory activating. The bronchial epithelium performs an important role in the response to pathogens and cigarette smoke, with production of proinflammatory cytokines (fig. 11) (Ling Ye et al., 2009).

In normal subjects, the bronchial epithelium show relatively low expression of ICAM-1 (adhesion molecule), NF-kB (nuclear transcription factor), STAT-4 (signal transducer and activator of transcription) and IL-8 (proinflammatory chemokine). In the lamina propria the number of T lymphocytes and macrophages is relatively low and can occasionally be observed neutrophils and eosinophils. The presence of activated T cells that express NF-kB, T cells and macrophages that express STAT-4 and IFNγ as well as activated neutrophils that express MPO (peroxidases to bactericidal activity) is relatively low.

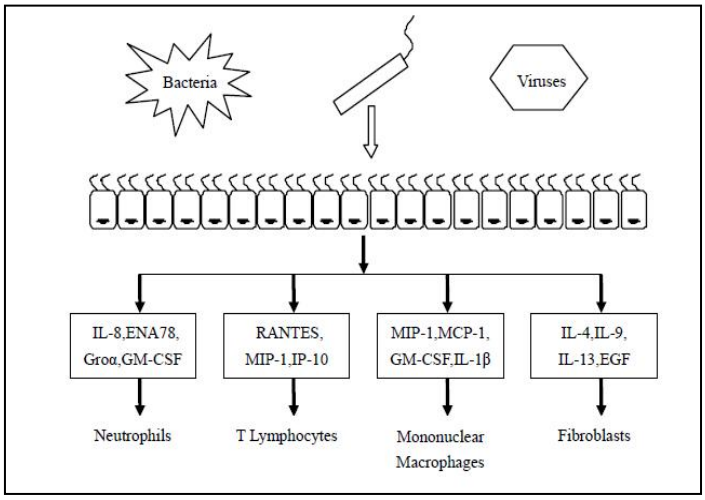


Fig. 11: Cigarette smoking and pathogens acting on the epithelium of the airways, stimulating cells to release pro-inflammatory mediators that act by activating inflammatory cells

In smokers with normal lung function, the epithelium shows similar characteristics to that of normal subjects. In the lamina propria the number of CD8 + T cells is increased but their activation status is unchanged compared to non-smokers. The NF-kB positive cells (including CD4 + and CD8 +) is increased concurrently with the increase infiltrate T cells. The endothelial activation (over-expression of ELAM-1) as well as the presence of activated neutrophils (MPO +) is in the range of normal subjects.

Individuals with mild to moderate COPD reveal an increase in the expression of ICAM-1, NF-kB, STAT-4 and IL-8. In lamina propria the number of CD8 + and CD68 + is increased as well as the number of activated T cells (both CD4 + and CD8 +) and

macrophages that express NF-kB, STAT-4 and IFN-γ. Endothelial ELAM-1 is increased in association with increased neutrophilia in the bronchoalveolar lavage (BAL) and sputum. The total number and activated neutrophils and eosinophils in bronchial tissue is slightly increased or unchanged. In case of severe disease, the bronchial epithelium shows an increase of expression of myeloperoxidase, ICAM-1 and IL-8. In the lamina propria the number of activated CD4 + and CD8 + decreased slightly while the number of macrophages and neutrophilsis higher. The IL-8 secreted from bronchial epithelium plays a critical role in addressing neutrophil and sustaining the chronic inflammatory state, characteristic of COPD.

1.3 Physiopathology

1.3.1 Airflow limitation and air trapping

The extent of inflammation, fibrosis, and luminal exudates in small airways correlates with the reduction of FEV and FEV / FVC (Hogg JC et al., 2004). The obstruction of the peripheral airways progressively traps air during expiration, resulting in hyperinflation (chronic accumulation of air in the lungs). Although emphysema is more associated with abnormalitygas exchange and the reduction of FEV, it contributes to air trapping during exhalation. This occurs in particular when the alveolar junctions of the small airways are destroyed, concomitantly with the worsening of the disease. Hyperinflation reduces inspiratory capacity, particularly during exercise (dynamic hyperinflation) and this results in dyspnea and reduced exercise capacity.

1.3.2 Gas exchange abnormalities

The gas exchange alterationsresult in hypoxemia and hypercapnia, and have different mechanisms in COPD. In general, the gas transfer worsens with disease progression. The severity of emphysema correlates with arterial PO2 and other markers of ventilation / perfusion (Rodriguez-Rosin R et al., 2009). Even the obstruction of peripheral airways causes the imbalance of ventilation / perfusion ratio and combining with the functional of the respiratory muscles, in severe disease, reduces ventilation and leads to carbon dioxide retention.

1.3.3 Mucus hypersecretion

The mucus hypersecretion, which determines the onset of chronic productive cough, is a typical aspect of chronic bronchitis and is not necessarily associated to the restriction to air flow. On the other hand, not all patients with symptomatic COPD show hypersecretion. When present, is derived from the respiratory epithelium metaplasia which leads to an increase in the number of goblet cells and increase the size of the submucosal glands in response tochronic airway irritation by cigarette smoke or other noxious agents. Several mediators and proteases stimulate mucus hypersecretion and

many of them exert their effects through the activation of the epidermal growth factor receptor (EGFR) (Burgel PL et al., 2004).

1.3.4 Pulmonary Hypertension

During the course of the disease, it can late develop from mild to moderate pulmonary hypertension. It is due to hypoxic vasoconstriction of small pulmonary arteries, which can cause structural changes that include intimal hyperplasia and, later, hypertrophy / hyperplasia of smooth muscle (Peinado et al., 2008). In vessels there is an inflammatory response similar to that seen in the airways and it is evident the dysfunction of endothelial cells. In emphysema, the loss of the pulmonary capillary bed may also contribute to increased pressure in the pulmonary circulation. Progressive pulmonary hypertension can lead to right ventricular hypertrophy and eventually to right heart failure

1.3.5 Systemic effects

It is increasingly recognized that COPD has many extrapulmonary effects, especially in patients with severe disease and that they have a major impact on survival and quality of life (Barnes PJ et al., 2009). Cachexia is commonly seen in patients with severe COPD. There may be loss of skeletal muscle mass and weakness as a result of increased apoptosis and / or muscle disuse. Patients with COPD have also more probability to have osteoporosis, depression and chronic anemia (Similowski et al., 2006). Increased concentrations of inflammatory mediators, can mediate some of these systemic effects. There is an increased risk for cardiovascular disease, which correlates with the increase in C-reactive protein (CRP) (Gan WQ et al., 2004).

1.3.6 Exacerbations in COPD

Exacerbations represent a further amplification of the inflammatory response in the airways of patients with COPD and may be triggered by infection with bacteria, viruses or by environmental pollutants. There is a relative lack of information about the inflammatory mechanisms involved in COPD exacerbations. Many of the inflammatory

mediators increase during exacerbations (Wedzicha JA et al., 2007): the IL-8 is one of the most inflammatory markers increased during exacerbations and correlates with the severity (WR Perera et al., 2007) together with the tumor necrosis factor alpha (TNF-α). During an exacerbation there is an increase of hyper-insuflation and air entrapment with airflow limitation, which explain the worsening of dyspnea (CM Parker et al., 2005). It also assists in a worsening of the alterations of ventilation / perfusion ratio resulting in severe hypoxemia. The number and frequency of exacerbations is associated with worsening of COPD over the years.

1.3.7 Bacterial infections

In normal conditions, the tracheobronchial tree and the pulmonary parenchyma represent a highly sterile environment, even though continuous exposure to microbial pathogens during breathing. Several studies have shown that in patients with COPD the living pathogenic bacteria flora is in actively proliferation (Monso E et al., 1999, Zalacain R et al., 1999).The molecules released by bacteria are potent inflammatory agents: include endotoxins, fragments of peptidoglycan, lipoproteins, microbial toxins and other molecules. These molecules evoke (in the site of infection) immune cells such as macrophages and neutrophils, activated TLRs and trigger the proinflammatory cytokines and chemokines release. In COPD is established chronic infection whose mechanisms are not yet entirely understand. Bacterial colonization causes chronic inflammation that leads to the destruction of the lung. "The hypothesis of the vicious circle" tries to explain this phenomenon (S Sethi et al., 2000): the weakening of the pulmonary immune defenses due to cigarette smoking allows the stable pathogens infection in the lower respiratory airways, and this further damages the immune defense systems and the cilia clearance of produced mucus, ultimately leading the destruction of the respiratory epithelium. In this way, the disease can be perpetuate itself.

Microbe	Role in exacerbations	Role in stable disease
Bacteria		
Haemophilus influenzae	20–30% of exacerbations	Major pathogen
Streptococcus pneumoniae	10–15% of exacerbations	Minor role
Moraxella catarrhalis	10–15% of exacerbations	Minor role
Pseudomonas aeruginosa	5–10% of exacerbations, prevalent in advanced disease	Likely important in advanced disease
Enterobacteriaceae	Isolated in advanced disease, pathogenic significance undefined	Undefined
Haemophilus haemolyticus	Isolated frequently, unlikely cause	Unlikely
Haemophilus parainfluenzae	Isolated frequently, unlikely cause	Unlikely
Staphylococcus aureus	Isolated infrequently, unlikely cause	Unlikely
Atypical bacteria		
Chlamydophila pneumoniae	3–5% of exacerbations	Commonly detected, pathogenic significance undefined
Mycoplasma pneumoniae	1–2% of exacerbations	Unlikely

Table 2: Pathogenic bacteria involved in acute and chronic infections of COPD.

Bacterial infections are not currently considered to be the major causes of disease development but are thought to have a major role in the frequent exacerbations that characterize the intermittent trend of COPD. Almost half of exacerbations are caused by bacterial infections, particularly by *H.influenzae, M.Catarrhalis, S.Pneuomaniae* and *S. aureus* (S Sethi et al., 2001) (table 2).

1.3.8 Role of the bacterial load

The bacteria pathogenicity and their role in exacerbations is explained by two models: according to the first model, the cause of exacerbations was due to the increase of pre-existing bacteria concentration, but at present, the empirical data do not seem to support this hypothesis (RA Stockley et al ., 2000).

A research study in 2007 allowed the evaluation of the difference in bacterial concentration in sputum of stable COPD subjects and exacerbate patients (S Sethi et al., 2007). The analysis focused on the research of the major pathogens involved in COPD exacerbations like *H.influenzae, M.catarrhalis* and *P.aeruginosa*. None of these seem to have a higher concentration in the sputum ofexacerbatepatients than stable COPD subjects. However, the bacterial load does not seem to be completely irrelevant: there is indeed a correlation between the concentration of bacteria in sputum and the intensity of airway neutrophilic inflammation (AT Hill et al., 2000); also in exacerbation COPD patients there is a significant increase in bacterial concentration compared to healthy subjects and patients with stable COPD (A Rosell et al., 2005).

1.3.9 Newbacterial strains typing

The second model that explains the role of bacterial infections in exacerbations involves the acquisition of new bacterial strains from the environment (Veeramachaneni SB et al., 2006). The host uses adaptive response systems toward the bacterial strain and, together with the use of antibiotics, controls and eliminates the pathogen. The adaptive response does not defend the host against new infections by different strains that would cause so the establishment of a new inflammatory process and consequent exacerbations. Clinical evidence for this model comes from an American study of 2002 in which 50 patients with COPD were visited every month for about 8 years. At each visit was evaluated the clinical status of the patient and sampled the sputum for laboratory analysis. The study showed an increase in exacerbations frequency following the isolation of new bacterial strains in the sputum(Sethi S et al., 2002).

The infection by new bacteria strains leads to a peculiar inflammatory profile, when compared with exacerbations without new pathogenic lines: in the first case there is a substantial increase of neutrophilic markers (Sethi S et al., 2007).In general, in COPD patients, the bacterial populations present in the airways are characterized by less variability and greater presence of specific populations (Han et al., 2012). In most cases, infection are driven by *H.influenzae, S.pneuomaniae, M.catarrhalis* and *P.seruginosa*. In presence of these bacterial strains has been observed a significant increase of exacerbations risk.

1.4 The Immune System

1.4.1 Innate and Adaptive immunity

The human body defends itself against external pathogens with the immune system, consisting of two components: innate immunity and adaptive immunity. Both allow the recognition of molecules "non-self" and trigger a response that leads to the elimination of micro-organisms associated with these molecules (fig 12).

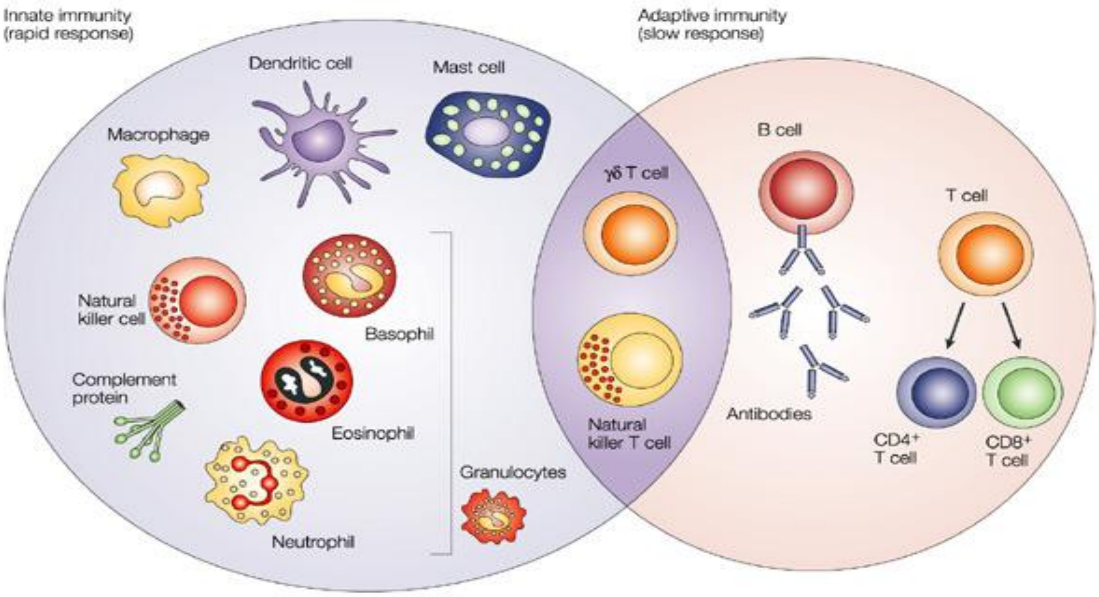


Fig. 12: innate and adaptive immunity cells

Innate immunity is the first defense form of the organism to be activated, it is not specific, and it does not have "memory"; the main components are:

• *physical and chemical barriers*: epithelia are the first tissues that come in contact with the microorganisms that can easily enter inside the body through the skin, the gastrointestinal tract and the respiratory tree by physical contact, ingestion and breathing.

In all epithelia, cells are in mutual contact and rest on a basement membrane that binds them to the underlying connective tissue, the *lamina propria*(fig.13). In addition to their role as a physical defense, epithelia secrete a wide variety of molecules which assist the

immune system: peptides, proteins and organic molecules including proinflammatory cytokines and chemokines with chemotactic action;

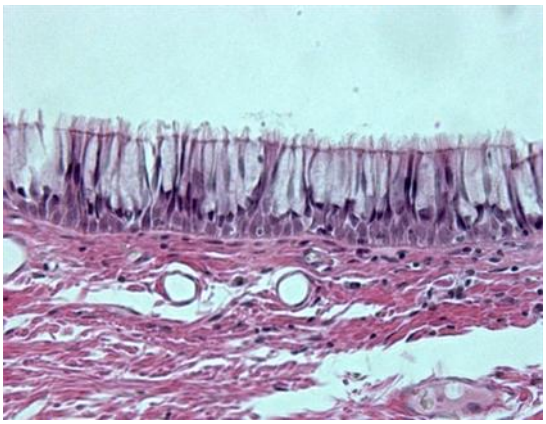


Fig. 13: Bronchial epithelium: it is possible to distinguish epithelial cells and thin basement membrane that separates the epithelium from the lamina propria, consisting of connective tissue

• cells with phagocytic activity:

-Neutrophils area subpopulation of polymorphonuclear leukocytes with phagocytic activity; together with basophils and eosinophils form the granulocytes, whose characteristic is to have the cytoplasm rich in granules. Neutrophils are the most widespread cells (50-60%) compared with all granulocytes. They have a peculiar nucleus containing from 3 to 5 lobes (fig.14). Neutrophils are short-lived cells (12-24 hours) and the percentage of circulating cells is only about 2% of the total; they normally reside in the bone marrow.

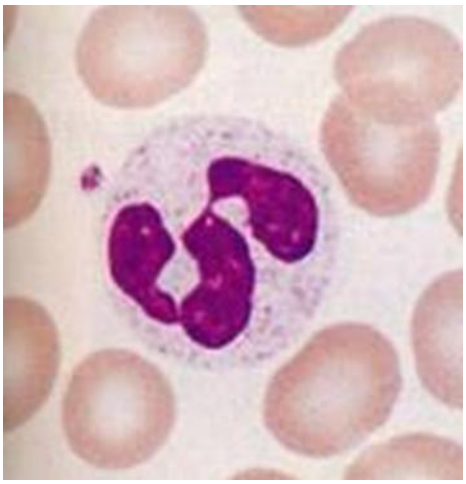


Fig. 14 Neutrophil: the cell in the figure contains a trilobal nucleus

The granules inside them are formed during differentiation by gemmation from the Golgi and are divided into three types: primary (or azurophilic), contain molecules with the function to kill and digest bacteria (eg. Elastase) and molecules with antibacterial activity (eg. defensins); secondary (or specific) contain lactoferrin to sequester iron and copper, lysozyme and metalloproteinases useful for neutrophil migration (digest the matrix Extracell) and tertiaries they contain gelatinase not associated with NGAL (Neutrophil gelatinase-associated lipocalin, present only in the secondary granules).

Neutrophils arrive at the site of infection in response to chemical stimuli with chemotactic action such as IL-8.Once in the bloodstream neutrophilsbind to endothelium by *selectins* (weak interaction) and thanks to the presence of chemokines are up-regulated *integrins* (such as Mac-1), which allow stable adhesion to endothelium and subsequent diapedesis. The extravasation occurs in four stages: rolling (reversible binding with endothelium), arrest, (irreversible binding), stable adhesion and diapedesis. The latter may be through the transcellular (through endothelial cells) or paracellular way (between an endothelial cell and the other); the matrix is traversed by the release of digestive molecules such as gelatinase, enclosed in the granules.

The pathogen may be killed intracellularly or extracellularly. In the first case, the neutrophil recognizes directly the pathogen PAMPs or recognize associated molecules such as antibodies or complement proteins.The pathogen is internalized inside a phagosome, which merges with the granules that release antimicrobial substances such as lysozyme and cathepsins.

-**Macrophages** are mononuclear leukocytes with phagocytic properties and are originate from circulating monocytes. Monocytes penetrate the tissue and differentiate into different types of macrophages. The macrophages activation occurs through two main types of program: *classical inflammatoryactivation*(M1), whose the principals activating stimuli are bacterial molecules (eg. LPS) or cytokines (eg. TNFa) and the *alternative activation* (M2), whose activating stimuli are anti-inflammatory cytokines (eg. IL-4 and IL-10, TGF-beta)(fig. 15). The M1 macrophages are called inflammatory macrophages. Theycan secrete pro-inflammatory cytokines and antimicrobial substances and are specialized in presenting antigen to cells of adaptive immunity. M2 macrophages are involved in the inflammation resolution and wound repair, then they can secrete anti-inflammatory cytokines such as IL-10 and TGF beta (from which however are activated).

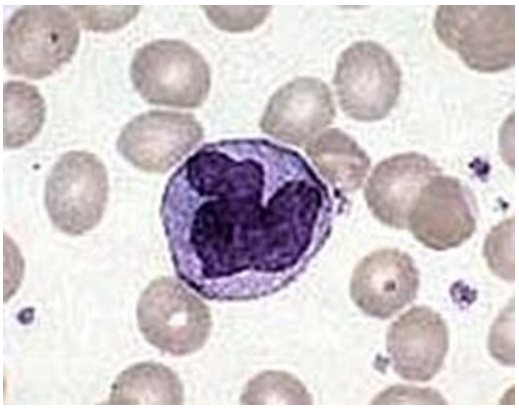


Fig. 15: Macrophage: macrophages represent differentiated cells from circulating monocytes

- **Natural Killer(NK)**: they represent a population of lymphocytes that are part of the innate immune system which, unlike the T and B lymphocytes, originate from the myeloid line in its differentiation process. The NK kill infected cells by pathogens through direct cytotoxic action and release pro-inflammatory cytokines including IFNγ (macrophages activator).
- **Basophils**: they are a polymorphonuclear leukocytes subpopulation,they are part of granulocytes and are the less representative cells of this class. They seem to be implicated in allergies and anti-parasitic response.
- **Eosinophils**: other subclass of granulocytes, particularly important in the responses to extracellular parasites such as helminthes.
- **Mast cells:** large immune cells, the main effector cells of immediate hypersensitivity reactions (allergy).
- **Dendritic cells**: characterized by thin cytoplasmic extensions, are defined as "sentinels of the immune system".They fulfill, together with B cells, the fundamental role of presenting antigen to T lymphocytes by modulating the activation.

The adaptive immunity is a much more evolved form of defense, whose strength and defensive ability are increase each subsequent exposure to the same pathogen. It implements highly specific mechanisms to a particular pathogen and is progressively refined to respond more effectively to the microorganism.

Its distinctive characteristics are: the extraordinary specificity for different antigens and the ability to "remember" (Immune Memory) and for this,to respond in

amore powerful way during repeated exposure to the same microbe. The word "acquired" emphasizes the fact that the extreme strength of the responses is reached through direct contact with pathogens. For his extraordinary ability to distinguish between different pathogens and macromolecules, sometime very similar, acquired immunity is often also called specific immunity.The components of the specific immunity are lymphocytes. B and T lymphocytes are distinguished: the first are develop in the bone marrow and are involved in antibody production. They constitute the humoral component of the immunity and they are normally active in extracellular pathogens elimination. The specific receptor of B lymphocytes is BCR (B-cell receptor) and it is constituted by a molecule of immunoglobulin IgD or IgM.

T cells are developed in the thymus and are a more heterogeneous group of adaptive immune cells: are divided into CD3+, CD4+ and CD8+, based on exposure in membrane of particular proteins defined cluster differentiations (CDs). CD3 is a protein complex associated with the T cell receptor, TCR. CD4 and CD8 are co-receptors whose in presence or absence allows to discriminate lymphocytes into helper T lymphocytes CD4+ and cytotoxic T lymphocytes CD8+. The CD4+ respond to the infected cells through the release of cytokines, while CD8 + implement a cytotoxic action against microorganisms.

1.4.2 Toll-like receptors

The receptors of the innate immune system, involved in the recognition of microbial antigens are called "Pattern Recognition Receptors" (PRRs) and the molecules recognized by them are called "Pathogen associated molecular patterns" (PAMPs), very conserved molecules among microbes, indicating the birth of ancient innate immune system. PRRs belong to the Toll-Like Receptors (TLRs), the RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs).

TLRs are trans-membrane glycoprotein, first identified in Drosophila melanogaster, which play an essential anti-parasitic role (B Lemaitre et al., 1996) and then characterized in humans. The N-terminal extracellular domain contains 16-28 motifs rich of leucine (LRRs, leucine rich repeats) long 24 amino acids each. The N-terminal extracellular domain determines the characteristic horse-shoe shape (Botos I et al. , 2011) and it is responsible for the recognition of the ligand. TLRs have all the

horse-shoe shape N-terminus with small curvature changes; the C-terminal cytoplasmic tail is characterized by the presence of the catalytic TIR domain (Toll IL-1 receptor) (JK Bell et al., 2003), named for the homology with the catalytic domains of proteins belonging to the IL-1 receptors family (LA O'Neill et al., 2007). The TIR domain is also present in some vegetal proteins involved in pathogens resistance, highlighting the ancient nature and role in immune responses, even preceding the divergence between plants and animals (TM Burch-Smith et al., 2007).

In mammals there are at least eleven TLR, each with different functions in antigen recognition of the innate immune system. Many of the ligands have been identified but others are still unknown (table 3). The ligand binding triggers a homo/heterodimerization which approaches the TIR domains of both subunits, allowing to call proteins involved in signal transduction (fig. 16). As shown in table 3, some receptors allow to create heterodimers that raise the microbial components spectrum recognized by TLRs.

Table 3: role of TLRs in the recognition of endogenous and exogenous ligands from Jennifer E. Cole et al, Mediators of Inflammation, 2010

TLR Receptor	Exogenous Ligand	Endogenous Ligand
TLR1	Mycoplasma tri-acyl lipopeptides [184], ngitides soluble factors (with TLR2) [185]	
TLR2	*Pam3CSK4* (synthetic TLR2/TLR1 agonist) [127], Mycobacterial lipoprotein (with TLR1) [190], Bacterial lipoproteins (with TLR6) [191, 192] Yeast carbohydrates, [192] Borrelia burgdorferi lipoprotein (with TLR1) [194] Staph epidermidis phenol-soluble modulin [196] Viral envelope glycoproteins [197, 198] *Peptidoglycan* (Gram + bacteria) [199] Glycoinositolphospholipids (Trypanozoma cruzi), Glycolipids (Treponema maltophilum), Porins (Neisseria), Zymosan (fungi), Atypical LPS (Leptospira interrogans and *Porphyromonas gingivalis*) [200–202]	Necrotic cells [186–189], *Apoliprotein CIII* [120], *Oxidised LDL* [36], Serum amyloid A [193], Amyloid beta [195] Versican [110],
TLR2/TLR4	HSP60 [203], *Chlamydia pneumoniae* [138], *HSP60 from Chlamydia pneumoniae* [106], *Porphyromonas gingivalis* [215]	*HSP60, HSP70, Gp96, HMGB1,* [204–211] *Hyaluronan fragment* [109, 212, 213] *Biglycan* [214]
TLR3	*Viral dsDNA* [48, 90, 216]	*mRNA* [217]
TLR3/TLR9	*CMV* [218, 219]	
TLR4	*Lipopolysaccharide,* [220–224] Viral envelope glycoproteins, [226, 227] Taxol (plant), RSV fusion protein, MMTV envelope proteins, [200] *HSP60 from Chlamydia pneumoniae* [93, 105]	Lung surfactant protein-A, [225] Tenascin C, [108] *Fibrinogen,* [228, 229] *Fibronectin EDA,* [230] *Heparan sulphate,* [231–233] *Beta-defensin 2,* [234] [235] *Minimally-modified LDL,* [113, 236] *Oxidised LDL,* [10] Amyloid beta peptide and oxididised LDL [115]
TLR5	Bacterial flagellin [237, 238]	
TLR6	Mycoplasma di-acyl lipopeptides [239], Group B Strep heat-labile soluble factor, Staph phenol-soluble modulin [200]	
TLR7	Various synthetic compounds including imidazoquinoline, loxoribine and bropirimine [200]	
TLR7/TLR8	Single stranded RNA [240–242]	
TLR7/TLR9		Nucleic acid-containing immune complexes [243–245]
TLR9	Hypomethylated CpG motifs in microbial DNA [241, 246, 247], *HSV-2* [241]	

TLR2 recognizes a variety of microbial components, including lipoproteins/lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid derived from gram positive (Werts et al., 2001). The great heterogeneity of ligands recognized by this receptor is explained by the ability of this receptor to form heterodimers with other TLRs as TLR1 and TLR6, both structurally related to TLR2. Furthermore, TLR2 can interact with proteins structurally not correlated such as, for example, the dectina-1, a receptor of the β-glucans, components of the fungal cell wall (BN Gantner et al., 2003).

TLR3 recognizes dsRNA, produced by many viruses during their replication cycle, and involves the production of type 1 interferons α and β, which are important in the antiviral response.

TLR5 is involved in the response to bacterial flagellin and it is an important receptor also present on epithelial cells of the lung mucosa (Hawn TR et al., 2003).

TLR7 and TLR8 are activated following the recognition of viral nucleic acids: ssRNA rich in uridine or guanosine present for example in the influenza virus (Hell F et al., 2004).

TLR9 is a receptor of CpG DNA (Hemmi H et al., 2000): the bacterial DNA containing motifs rich of un-methylated CpG that control an immune-stimulatory effect through the activation of TLR9.

TLR11 is the latest toll-like receptor identified, it is mediator of resistance ofuropatogenic bacteria in mice (D Zhang et al., 2004); it recognizes molecules of Toxoplasma profilin (F Plattner et al., 2008).

TLRs are differentiated for the different cellular localization: the TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed in the plasma membrane while the TLR3, TLR7, TLR8 and TLR9 are localized in the intracellular compartment in endo-lisosomi (fig. 15).

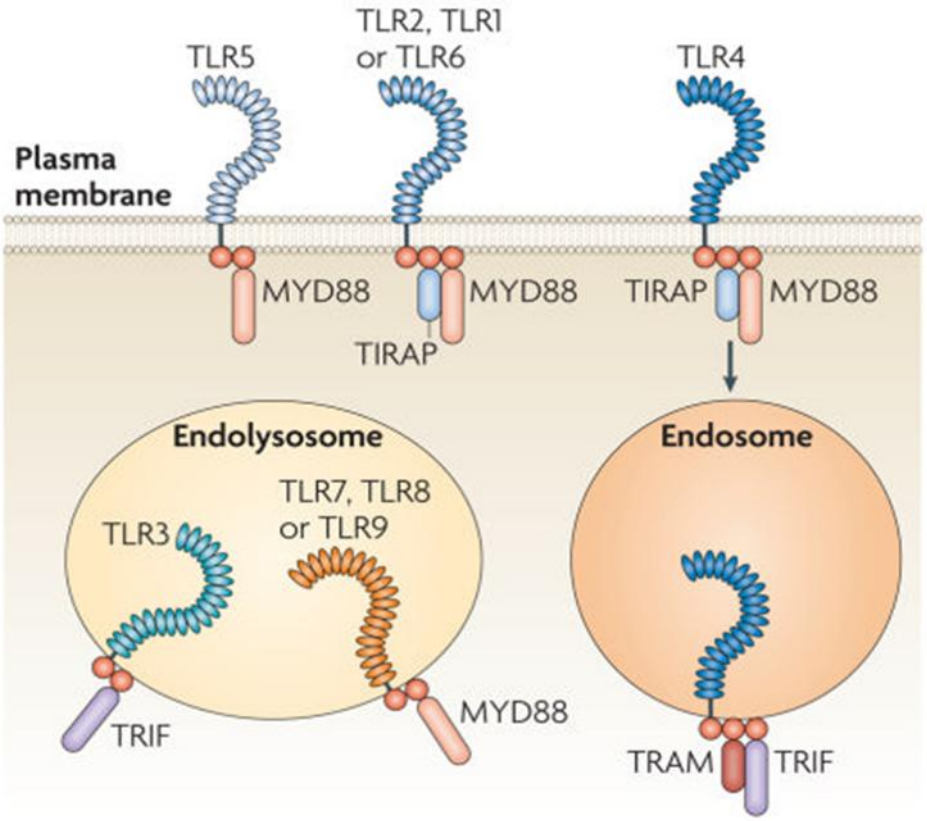


Fig. 16: Cellular localization of different Toll-Like Receptors

The surface TLRs bind molecules present predominantly in the extracellular space and the signal transduction is initiated directly at the membrane level; the localization is crucial for the activation: the inability in the transport of the protein to the cell surface can cause a block of signaling by the receptor (M Carty et al., 2006). Endo-lysosomial TLRs detect the presence of nucleic acids derived from viral or bacterial: in this case, in addition to a correct translocation of the receptor (Latz et al., 2004) it is necessary that the ligand molecules are internalized by the cell and transported into the lysosomal compartment where reside TLRs.It is still unclear how "intracellular" TLRs have access to their ligands. Numerous experiments on cell cultures have shown that the addition of ligands to the culture medium leads to TLRs activation by a mechanism dependent from endocytosis and from endosomal maturation process (H Hacker et al., 1998). Another proposed mechanism involves autophagy, a process by which the cell sequesters cytoplasmic material in vesicles called autophagosomes which fuse with lysosomes to form the autophagolysosomes (Z Xie et al., 2007).

1.4.3 Signal transduction

Stimulation of TLRs triggers the expression of genes involved in inflammatory responses: the pathways are preserved and leading to activation of transcription factors such as NF-kB (Nuclear Factor-kB), AP-1 (activating protein-1) and IRFs (IFN regulatory factors) (T Kawai et al., 2006). NF-kB is a heterodimeric transcription factor composed of subunits p65 and p50 (Karin M et al., 2005). In the absence of stimulus NF-kB is sequestered in the cytoplasm in an inactive form through interaction with protein inhibitors of NF-kB (IkBs). Following stimulation, the IkBs are phosphorylated on serine residues by a complex formed by two IKK kinases, IKKα and IKKβ, and a regulator, IKKγ / NEMO. The phosphorylation leads to the ubiquitination and subsequent degradation of IkBs by the proteasome; NF-kB is then released and can enter the nucleus where it binds to the kB site mediating the expression of target genes. AP-1 is a generic term for a homo or heterodimer formed by different combinations of transcription factors as Jun, Fos and ATF, that binding DNA at the same target site, the AP-1 site, mediating the expression or silencing of target genes (Shaulian et al., 2001). The regulatory factors IRFs are proteins sequestered in the cytoplasm in the absence of stimulation; following binding ligand-receptor, are phosphorylated by kinases IKKs, including TBK1 (TANK-binding kinase 1) and Ikki, and translocate to the nucleus where they regulate the expression of target genes (S Sharma et al., 2003).

The recognition of the ligand by TLRs triggers their dimerization and the triggering of the pathway originates following the interaction between the TIR intracellular domain of TLRs and the TIR domain of cytosolic molecules (adapters):

- Myd88 (myeloid differentiation primary response protein 88)
- TIRAP (TIR domain-containing adapter protein) / Mal (Myd88 adapter-like)
- Trif (TIR domain-containing adapter inducing IFNβ) (also called TICAM1)
- TRAM (Trif-related adapter molecule (also called TICAM2)

TIRAP and TRAM are sorting adapters that recruit respectively Myd88 and Trif, signaling adapters. What differentiates the various TLRs is the use of differential

adapters: as shown in figure 17 each TLR uses a unique combination of adapters which leads to activation of transcription factors mentioned above.

The main use of Myd88 as adapter, except TLR3 that exploits instead exclusively TRIF, allowed to distinguish two ways of signaling: the Myd88 dependent pathway and Myd88independent pathway (Trif pathway dependent). It is interesting that all the endosomal receptors perform a signal transduction independent of TIRAP, suggesting a correlation between the localization of TLRs and the use of specific adapters.

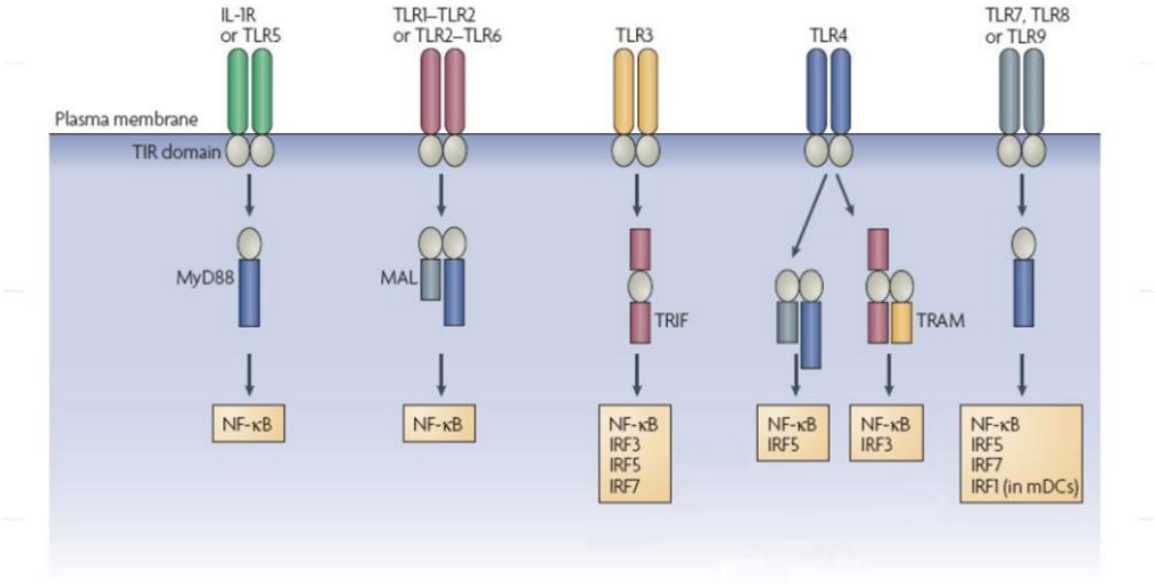


Fig. 17: The differential use of the adapters leads to different pattern of gene expression: for example, the activation of TLR3 and TLR4 induces the expression of type I interferons (IFNs) to the contrary of TLR2 and TLR5. Also TLR7, TLR8 and TLR9 leads to induction of IFNs but with different mechanisms than TLR3 and TLR4.

1.4.4 Myd88-dependent signaling pathway

It is a signaling pathway used by all TLRs except TLR3. It is so called because it uses the Myd88 as central adapter. The interaction between the intracellular TIR domains of TLRs and adapter TIRAP, also known as Mal (Myd88 adapter-like) allows the recruitment of Myd88. TIRAP is a protein containing a C-terminal TIR domain and it is essential for the signaling pathway mediated by Myd88: knockout mice TIRAP/Mal

show a phenotype very similar to Myd88 knockout mice, characterized by a strong inability to produce proinflammatory cytokines (Horng T et al., 2002).

However, despite the almost total inability to produce inflammatory cytokines, it was observed that in the absence of Myd88 and TIRAP, is activated NF-kB, although in a weak way, following exposure to TLR4 ligands; which does not happen instead using ligands of TLR2 (Yamamoto M et al., 2002). The late activation of NF-kB, together with the expression of genes regulated by IRFs in the absence of TIRAP and Myd88 and in the presence of TLR4 agonists is compatible with the hypothesis of activating a Myd88 independent pathway, which exploits other adapters different from TIRAP and Myd88: TLRs not using Myd88, such as TLR3, TLR5, TLR 7 and TLR8, showed a normal activity in knockout mice for TIRAP, highlighting the primary importance upstream role of TIRAP in Myd88 dependent pathway.Myd88, recruited through TIRAP, binds proteins of IRAK (interleukin-1 receptor-associated kinase)family, initially identified as partners signaling of IL-1R. Now are known four IRAK, all with a death domain in the N-terminal, through which they interact with Myd88: IRAK1, IRAK2, IRAK4 and IRAK-M. IRAK1 and IRAK4 have kinase activity due to an aspartate residue in the kinase domain, important in signaling mediated by Myd88. Aspartate is absent in IRAK-4 and IRAK-M (Janssens S et al., 2003), making them devoid of kinase activity and suggesting a role as negative regulators of signal transduction.Studies on KO mice corroborate this thesis: the IRAK1knock-out causes a weak response on LPS stimulation (Swantek JL et al., 2000) as well as the absence of IRAK4 (N Suzuki et al., 2002). The IRAK-M knock-out, in contrast, reveals an increase in the production of proinflammatory cytokines in response to different ligands of TLRs (Kobayashi K. et al., 2002), supporting the hypothesis of a role as a negative regulator. A study of the 2002 demonstrated that IRAK4 acts upstream of IRAK1 allowing activation by phosphorylation (Li S. et al., 2002) and IRAK-M would be responsible to the inhibition of IRAK4/IRAK1 dissociationfrom Myd88, blocking the signaling process (Kobayashi K et al., 2002).IRAK1 activated can associate with TRAF6 (tumor necrosis factor receptor-associated factor _6), a protein that contains two TRAF domains at the C-terminal (TRAF-N and TRAF-C), which allow interaction with other TRAF proteins, and a N-terminal RING domain, typical of many E3 ubiquitin-ligase, through which forms a complex with Ubc13 and UEV 1A for poly-ubiquitination of target proteins at the level of lysine 63 (K63), activation signal for many signaling proteins (Chen ZJ, 2005). TRAF6 activates TAK1 (transforming growth-factor-β-

activated protein kinase 1), a member of the family of MAP kinase kinase kinase (MAPKKK). TAK1 forms a complex with TAB1, TAB2 and TAB3 thanks to zinc-finger domains that recognize ubiquitin chains on lysine 63 of TAK1 (ZJ Chen, 2005). The complex TAK1/TAB activates the IKK complex that induces the phosphorylation and subsequent degradation of IkBs (inhibitors of NF-kB), allowing the activation of NF-kB and its translocation to the nucleus, where it induces the expression of inflammatory cytokines (Wang C et al., 2001). Parallel with IKK activation, TAK1 phosphorylates two family members of the MAP kinase kinase, MKK3 and MKK6 (Wang C et al., 2001), which activate the MAP kinases JNK and p38. Even ERK is activated in response to different ligands of TLRs by MEK1 and MEK2, but the upstream way that leads to phosphorylation of MEK1 and MEK2 is not yet known. Fibroblasts and B cells harvested from KO mice for TAK1 show a reduced activation of NF-kB, JNK, p38 and ERK in response to agonists of TLRs (Sato S et al., 2005) corroborating its primary role in the Myd88 dependent signaling pathway (fig. 18).

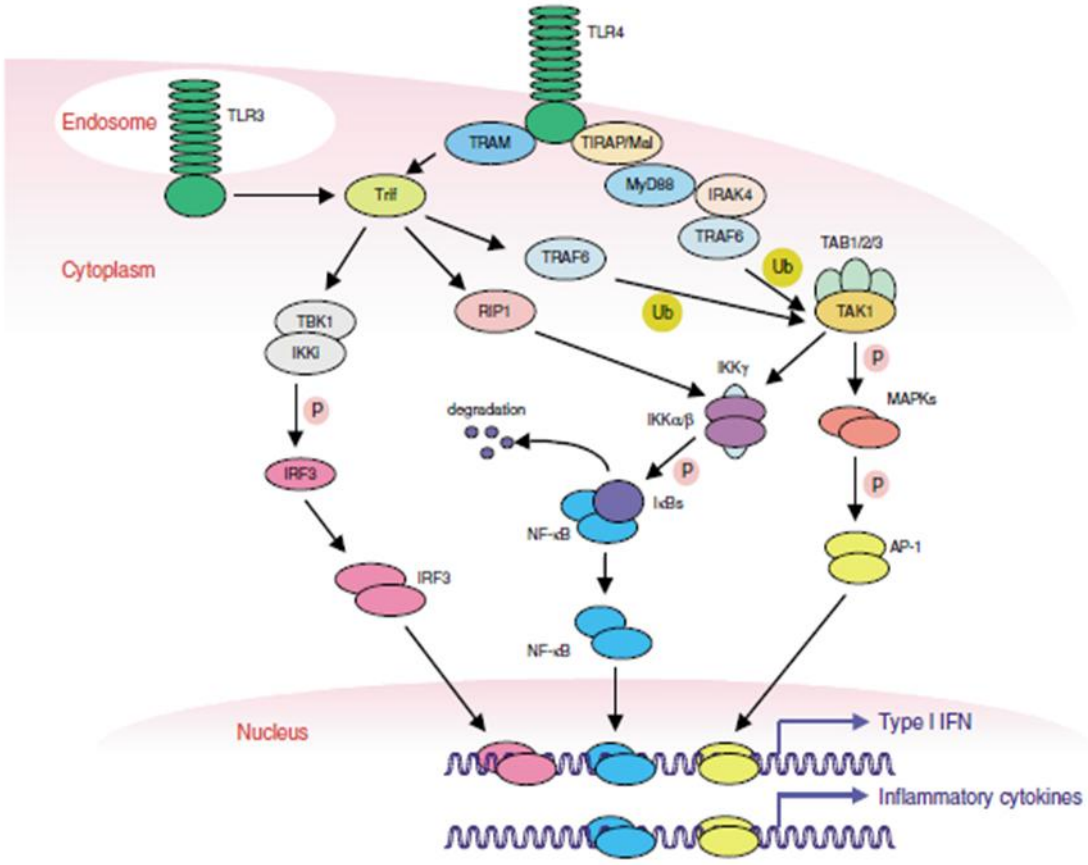


Fig. 18: Signalling pathways used by TLRs: shown the TLR4 as unique among all TLRs to transduce the signal via TRAM and Trif adapters (Myd88 independent pathway) or through TIRAP/Mal and Myd88 (Myd88 dependent pathway).

1.4.5 Myd88-independent signaling pathway

As mentioned above, cells derived from knockout mice for Myd88 do not produce pro-inflammatory cytokines in response to TLRs ligands. NF-kB and JNK are not activated using agonists of TLR2, TLR5, TLR7 and TLR9 suggesting the use only of the Myd88 adapter by these (Hemmi H et al., 2002) (F Hayashi et al., 2001). However, LPS is able to mediate the activation of NF-kB and MAP kinase in the absence of Myd88 with a slower kinetic than cells where Myd88 is present (Kawai T et al., 1999). In addition LPS induces the production of type I interferons, especially IFNβ (Theofilopoulos AN et al., 2005). The TLR3 does not use Myd88 and its activation, following the recognition of dsRNAs, leads to the expression of IFNβ (Alexopoulou L et al., 2001). These data suggested the existence of a second TLR4 signaling pathway associated with the activation of the IRF factors (interferon regulatory factor) with subsequent production of antiviral type 1 interferons, which uses other adapters by Myd88.

A central adapter in Myd88 independent signaling pathway of TLR4 is Trif.Further the activation of NF-kB and MAP kinase, this pathways also enables to produce IFNβ (Oshiumi H et al., 2003). In KO mice for TRIF is not activated IRF3 and there is no production of IFNβ in response to LPS, while the early activation of NF-kB and MAPK kinase is not altered (Diebold SS et al., 2003). However, both pathways are required to have a maximum inflammatory response after LPS stimulation.Trif, as Myd88, does not interact directly with TLR4, but needs an adapter called TRAM (Trif-related adapter molecule) (M Yamamoto et al., 2003). Trif has a pattern Rhim (Rip homotypic interation motif), necessary for the activation of NF-kB, which allows interaction with proteins of RIP (receptor interacting protein) family (E Meylan et al., 2004). Interaction Trif-RIP1 is responsible for the activation of NF-kB but RIP3 is a negative regulator of this pathway by blocking the link between Trif and RIP1 (Meylan E et al., 2004).The activation of NF-kB by Trif can also follow other paths: the TRAF6 binding domains present in the N-terminus of Trif allow its interaction with TRAF6, leading TAK1 activation by ubiquitination on lysine 63. Trif mutated in the TRAF6 binding site is unable to activating NF-kB (Sato S et al., 2003). This two pathways, Trif-TIP1 and Trif-TRAF6 converge in the activation of the IKK complex that induces the phosphorylation and subsequent degradation of IkBs, allowing the activation of NF-kB.

As discussed above, the Myd88 independent pathway (Trif-dependent) also brings up-regulation of the gene encoding the type 1 interferons, especially IFNβ, whose transcription is controlled by several factors including IRF3 and IRF7 (Theofilopoulos AN et al., 2005). These last normally reside in the cytoplasm in an inactive form but in response to LPS are phosphorylated by IKKS kinases, different from those involved in the activation of NF-kB (IKKα and IKKβ): TBK1 (TANK-binding kinase 1) and Ikki.The phosphorylation allows IRFs to enter in the nucleus and regulate the expression of genes, including the coding for IFNβ (Sharma S et al., 2003). IRF3 is constitutively expressed while IRF7 is induced by LPS stimulation or viral infections. The initial induction depends more on IRF3: the IFNβ released from stimulated cells, that have activated IRF3, acts on adjacent cells and induces the expression of IRF7 by the JAK-STAT pathway mediated by IFN1receptor, leading to an amplification of the antiviral response through a positive feedback mechanism (T Kawai et al., 2006) (fig. 19).

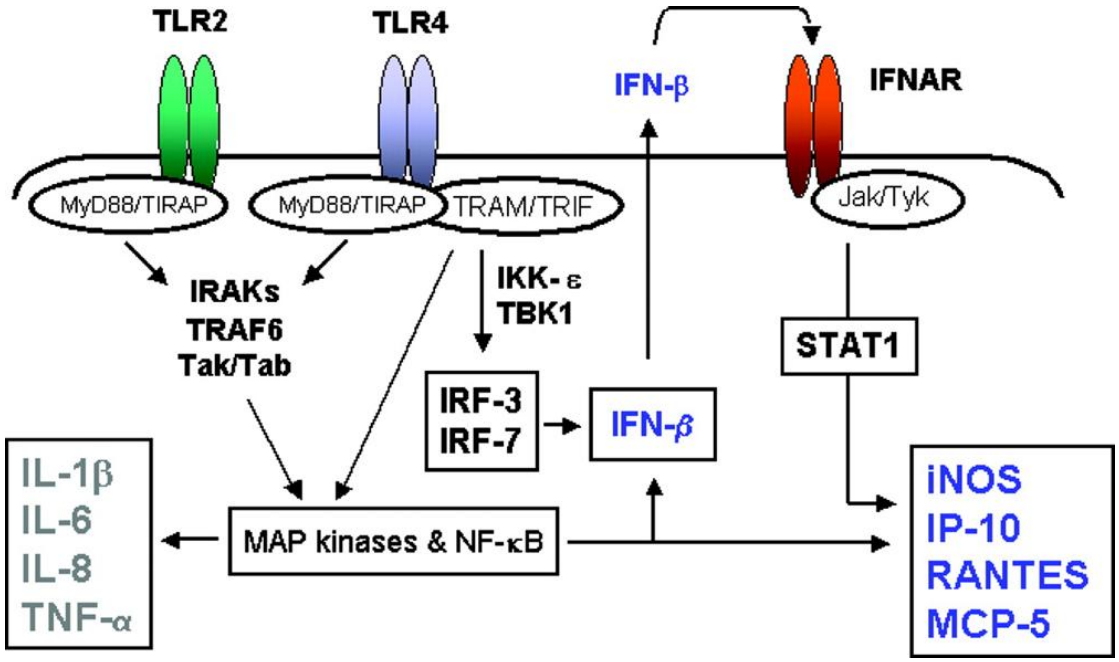


Fig. 19: The Myd88 dependent signaling pathway is used by all TLRs, with the exception of TLR3, and leads the activation of MAP kinase and NF-kB with final production of pro-inflammatory cytokines including IL-1β and TNFα and cytokines with chemoattractive activity such as IL-8. The Myd88-independent pathway is used by TLR4 to produce IFNβ following the activation of the IRFs factors. It is also shown the positive feedback process that leads the amplificationof the antiviral response: interferon released from stimulated cells acting by paracrine and autocrine signal onIFNs receptor (IFNAR), allowing the activation of INOS with nitric oxide production and chemoattractive molecules such as IP-10 (interferon-gamma-inducible protein 10).

1.4.6 MAP kinase

Map kinase (MAPK, mitogen-activated protein kinase) are a family of proteins involved in the cellular response to external stress.They participate in several pathways involved in embryogenesis, cell differentiation, cell proliferation (G Pearson et al., 2001) and are involved in the signaling of TLRs with production of pro-inflammatory cytokines in both Myd88 dependent and in Trif-dependent signaling pathway. There are three subfamilies of MAPKs: ERKs (extracellular signal-regulated kinases), JNK (c-jun N-terminal kinases) and p38 kinase (Zarubin T et al., 2005).They are regulated by phosphorylation cascades mediated by two more protein kinases, the MAPKKK and MAPKK. In every known pathways, kinases immediately upstream of the MAPK are proteins of MEK/MKK (MAP/ERK kinase) family, enzymes that phosphorylate serine, threonine and tyrosine in target MAPK (Kosako H et al., 1992). In turn, the MEK proteins are activated by MEKKs (MEK kinases) by phosphorylation on serine and threonine residues in their activation domain (Zheng CF et al., 1994).The phosphorylation cascade as a signaling mechanism allowsthe MAPK to receive inputs from multiple different pathways to suppress or enhance the signal (Frost JA et al., 1997). In addition the signal is amplified because the downstream kinases are normally more abundant than the upstream (ErredeB et al., 1995).

In the signaling pathways of TLRs are involved the MAPK JNK, p38 and ERK. The first two participate both the Myd88 dependent pathway and Myd88 independent pathway: the first kinase that intervene is TAK1, a MEKK activated by ubiquitination on lysine 63 (K63) by TRAF6 conjugated in Ubc13 and Uev1A.TAK1activated interacts with TABs proteinsand phosphorylates two different MAPKK, MKK3 and MKK6, which phosphorylate and activate p38 MAPK and JNK. ERK is activated by MEK1 and MEK2 kinases with not yet knownmechanisms. JNK and p38 play an important role in the activation of the transcription factor AP-1, which regulates the expression of pro-inflammatory cytokines.

MAPK p38 are a heterogeneous group of proteins composed by p38α, p38β, p38γ and p38δ.The most important isoform that participates in TLRs cascade is p38α (also called p38): p38 can also be activated after auto-phosphorylation through interaction with the TAB1protein (Ge B, et al., 2002). However, using KO cellsfor MKK3 and MKK6 has been shown that the production of pro-inflammatory cytokines

occurs exclusively in the presence of MKKs (Brancho D et al., 2003).The activation of p38 plays an essential role in the production of pro-inflammatory cytokines including TNFα and chemokines such as IL-8 (Zarubin T et al., 2005) and many chronic inflammatory diseases such as rheumatoid arthritis or IBD (Inflammatory Bowel Diseases) are associated with an up-regulation of this MAPK (Hollenbach E et al., 2004).

MAPKs proteins are encoded by three different genes: Jnk1, Jnk2 and Jnk3.Thesekinases can be phosphorylated not only by MKK3 and MKK6, but they are phosphorylated mostly by two more MAPKKs, MKK4 and MKK7, and together with p38 activate AP-1, favoring the production of pro-inflammatory cytokines (RJ Davis, 2000) (fig. 20)

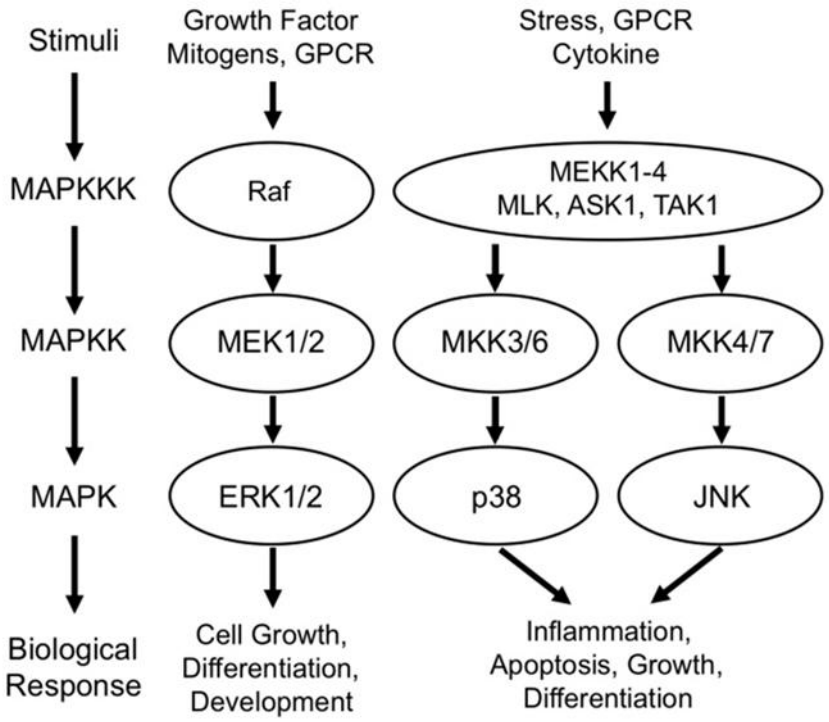


Fig. 20: MAPK are proteins involved in various cellular processes in response to extracellular stimuli: the MAPK signaling cascade passes through the upstream activation by two other kinases calls MAPKKK and MAPKK.

1.4.7 TLR4

Among all the Toll-like receptors, TLR4 is the receptor that most of all attracted the attention of researchers, for its ability to be activated in response to many bacterial

infections and its peculiar mechanism of signal transduction mediated by both Myd88 and Trif. Furthermore, in contrast to other TLRs, TLR4 is the only one that does not directly binds its ligand (LPS), but requires another protein, MD2, as well as extracellular LPS chaperones, LBP and CD14 (R Jerala , 2007).

1.4.8 LPS

The LPS (lipopolysaccharide, bacterial endotoxin) is one of the most powerful PAMPs able to activate an immune response in the host and it is recognized by a receptor complex formed by the TLR4 and associated molecules with it (Beutler B, et al., 2003).

LPS is the major component of the outer membrane of gram-negative bacteria and for its basic function is a molecule whose general structure is highly conserved among gram-negative bacteria. It is a glycolipid divided into 3 characteristics regions: the **lipid A**, the **core polysaccharide** and the**O-antigen** regions (fig.21) (BS Park et al., 2009).

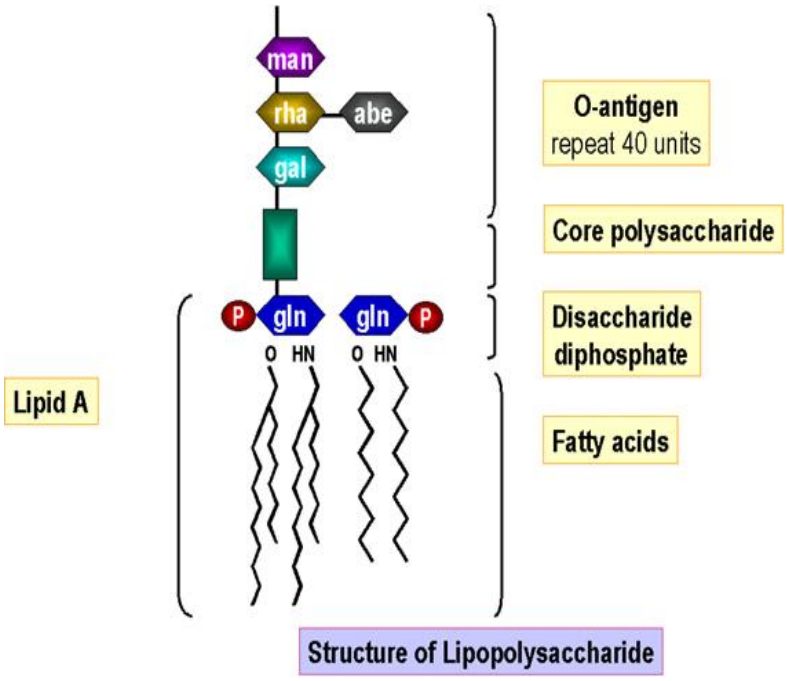


FIG 21 General structure of the lipopolysaccharide. The molecule contains three main regions: lipid A, the LPS anchorage area to the bacterial wall, the central core and the outer O-antigen, which is the component most variable among all the bacterial strains gram negative

Although the general structure is almost the same, the LPS shows variability between different bacterial strains. In order to allow a response to a wide spectrum of gram-negative bacteria, the receptor complex of TLR4 recognizes the most conserved LPS region, lipid A; despite this, variations in core and O-antigen regions may affect response and signaling pathways (Z Jiang et al., 2005). The lipid A is constituted by a molecule of the esterified and phosphorylated

glucosamine, conjugated to long chain fatty acids; it represents the more hydrophobic portion of the molecule and it can binds to the outer membrane of the bacterial cell wall: it constitutes the real toxin.

The core polysaccharide is the central part of the molecule and is divided in inner core and outer core: the first is the region closest to the lipid A and is constituted by the chetodeossioctonic acid and a heptose sugar, an atypical sugars fundamental for bacteria and therefore highly conserved between all the bacterial species. The outer core is relatively more variable and is formed from sugars with six common carbon atoms. O-antigen is instead formed by a long polysaccharide chain whose monosaccharides components vary between different bacteria strains. There are at least 20 different types of sugars, many of which are dideossihexoses that are found in nature only on gram-negative bacteria wall. O-antigen represents the most variable portion of the LPS and confers resistance to phagocytosis.

1.4.9 Receptor Complex

LPS is an amphipathic molecule that tries to aggregate: TLR4 is not able to bind it directly but it needs accessory proteins with which it forms a receptor complex. The physical recognition of lipopolysaccharide is mediated by TLR4 complexed to a co-receptor called MD2 (myeloid differentiation protein 2) (R Shimazu et al., 1999). The extracellular domain of TLR4 has the characteristic morphology of horseshoe typical of TLRs due to the presence of LRRs motifs rich in leucine;MD2 has a β-cup structure that forms a large hydrophobic pocket within which the ligand is bound (U Ohto et al., 2007). LPS is extracted from the bacterial membrane and transferred to the complex TLR4-MD2 by two other accessory proteins: LBP (LPS-binding protein) and CD14 (Miyake K, 2006) (fig.22). LBP binds LPS aggregates and transfers a monomer of LPS to CD14; the latter is required to transfer the monomer to MD2. The formation of the trimer LPS- MD2-TLR4 is the final event of the LPS extracellular recognition (Jerala R, 2007).

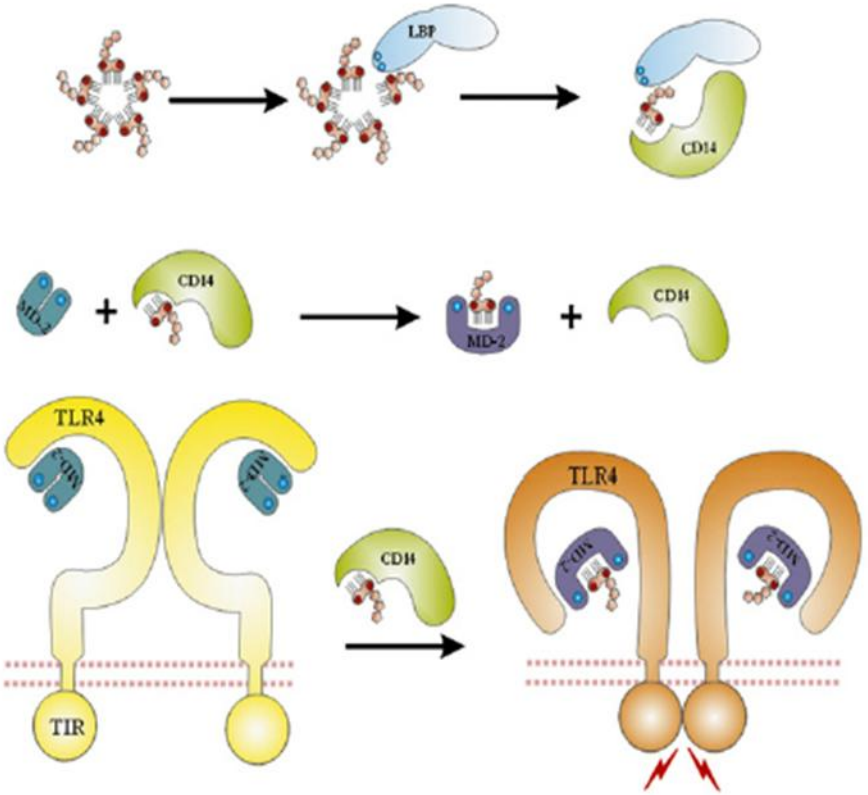


Fig.22: The TLR4 receptor complex which mediates recognition of bacterial lipopolysaccharide is formed by two accessory molecules, LBP and CD14.These molecules have the task to "present" the LPS to the complex formed by TLR4 and MD2.

LBP is a glycoprotein belonging to the families of the *lipid transfer/LBP* (LT/LBP), which also includes BPI (bactericidal/permeability-increasing protein), CETP (cholesteryl ester-binding protein) and PLTP (phospholipid transfer protein); it is mainly produced by hepatocytes but also from muscle and epithelial cells (Zweigner J et al., 2006).The N-terminal domain of the protein has a series of cationic residues essential for binding to LPS (Lamping N et al., 1996) while the C-terminal domain allows the binding to CD14 (RR Schumann et al., 1997). At low concentrations of LPS, LPB enhances the response by extracting LPS from bacterial membranes (Vesy CJ et al., 2000) and presenting it to CD14, whereas at high concentrations inhibits the response transferring the LPS to serum lipoproteins forming aggregates with LPS (Gustmann T et al., 2001).

CD14 is a glycoprotein present both in soluble form (sCD14) and anchored to the membrane by GPI (mCD14) and is essential for the response to low concentrations of LPS (Wright SD et al., 1990).As well as accessory molecule for the response of TLR4 to LPS,CD14 supports the signal of TLR1, TLR2 and TLR6 in response to

lipopeptides, LTA, peptidoglycan and also of TLR3 in response to dsRNA (HK Lee et al., 2006). The LPS binding site is present at the level of the N-terminal domain of the protein (Stelter F et al., 1997) where a cluster of positively charged residues form a large hydrophobic pocket that allows to accommodate the lipid A. In addition, CD14 can bind saccharide chains of LPS and peptidoglycan (Dziarski R et al., 2003).

The CD14 function is not limited to simple chaperone for LPS recognition by TLR4-MD2 but participates in Myd88-independent signaling pathway. CD14 controls the endocytosis of TLR4, key event for the activation of IRF-dependent pathway leading to the production of IRF3 (I Zanoni et al., 2011).

MD2 is a protein that belongs to the ML superfamily, a group of proteins that bind lipids (Inohara N et al., 2002). MD2 has been identified as unique accessory protein strictly necessary for the response to LPS: in KO mice for TLR4 or MD2, researchers reveal the same phenotype not responsive to LPS (Hoshino K et al., 1999; Nagai Y et al., 2002).

MD2 has the capacity to form oligomers through covalent bonds but it has been shown that only monomeric form is capable of binding monomers of LPS and to allow the signaling ofTLR4 (Visintin A et al., 2001); the analysis of the primary structure has revealed the presence of 7 cysteines involved in the formation of three intramolecular disulfide bonds necessary for the correct functioning of the protein. This it is demonstrated by the absence of activity in case of mutations (Mullen GE et al., 2003).

MD2 has a β-cup structure that forms a large hydrophobic pocket, thanks to the presence of cationic residues, inside of which is bound the ligand (Ohto U.et al., 2007);contrary to LBP and CD14, it is not able to bind other lipid molecules differ from LPS.MD2 exists in a soluble form that is anchored at the TLR4 membrane: the complex TLR4-MD2 binds LPS with a greater affinity than MD2 soluble alone (Akashi S et al., 2003), however, the ability of binding to TLR4 does not vary between MD2 and MD2-LPS (Visintin A et al., 2005). The binding of lipid A to MD2 allows dimerization of the TLR4 with association of intracellular TIR domain and the subsequent recruitment of sorting (TIRAP and TRAM) and signaling (Myd88 and Trif) adapters (fig. 21).

As described above, the pathways that lead to the production of pro-inflammatory cytokines following the binding with LPS involve the use of different adapters. TLR4 is the only one of all TLRs to use all 4 adapters Myd88, TIRAP, Trif and TRAM: the first two are involved in the Myd88-dependent signaling pathway with production of pro-inflammatory cytokines and chemokines and the last two in the

Myd88-independent signaling pathway (Trif-dependent) with production of type I interferons by activation of IRF3.The cellular localization of TLRs play a key role on their signaling pathways: endosomal TLRs (TLR7, TLR8, TLR9) for example perform a signal transduction independent of TIRAP but only using Myd88: the Myd88-dependent signaling pathwaycan then be performed from different cellular compartments (Kagan CJ et al., 2006).Contrary to what has been thought in the pastthe internalisation of TLRs does not abolish the ability to respond to ligands (Husebye H et al., 2006), but rather increases the possibility of activating specific signaling pathways (Kagan CJ et al., 2008).The activated TLR4receptor complex is localized in a well-defined region of the plasma membrane, the lipid rafts (Triantafilou M et al., 2004). These last are glycoprotein microdomains characterized by high amounts of cholesterol and sphingolipids compared to the rest of the membrane and are very rich in PIP2 (phosphatidylinositol 4,5-bisphosphate): they can bind several proteins, including GPI-anchored proteins (CD14), transmembrane proteins (TLR4) and miristilate protein (TRAM).

TIRAP is the sorting adapter that allows to recruit Myd88 and it is activated in lipid rafts through a binding domain to PIP2 in a process mediated by ARF6 (ADP ribosylation factor 6), positive regulator of production of PIP2(Kagan CJ et al., 2006). The binding capacity of TIRAP to PIP2 can explain why this adapter is used by TLR2 and TLR4, both located in the membrane (where there is a high concentration of PIP2) and not by TLR3, TLR7 and TLR9 residing in cellular compartments, where is not present PIP2. Myd88 recruited by TIRAP leads to "premature" activation of NF-kB and AP-1 in Myd88-dependent pathway.

The second signaling pathway, Trif-dependent, is activated after an internalization process of the receptor complex in a process mediated by dynamin (Husebye H et al., 2006): dynamin is a GTPase protein essential for the formation of vesicles from the plasma membrane and for the turnover of the endosomes. Inhibition experiments of dynamin (using a specific inhibitor called dynasore) showed that in its absence, TLR4 does not internalize and not activated IRF-3 with no production of IFNβ (Kagan CJ et al., 2008). Another newly discovered protein, essential for the internalization of TLR4 is CD14, considered by many years as a molecule designed solely for LPS trafficking: it has been shown that in the absence of CD14 the Trif-dependent pathway in TLR4 is not activated because of the impossibility by the cell to internalize the receptor (I Zanoni et al., 2011).In the membrane, the complex resides in

a region rich in PIP2 and signals "preferentially" by Myd88 through interaction of TIRAP and TLR4. During endocytosis, the concentrations of PIP2 drop dramatically (Botelho RJ et al., 2000) leading to the release of TIRAP-Myd88. The TIR free domain is now bound by TRAM that, by analogy with TIRAP, is the sorting adapter deputy to the recruitment of Trif: it is then activated the Trif-dependent pathway that lead to the production of type I interferons and activation "late "of NF-kB and AP-1 (Kagan CJ et al., 2008) (fig. 23).

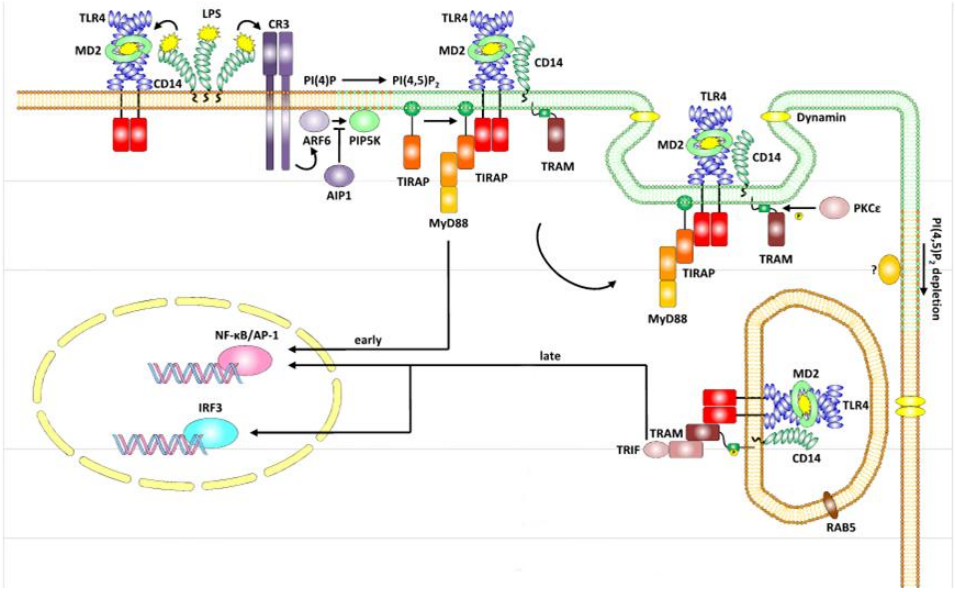


Fig. 23: TLR4 signaling pathways: LPS is recognized and transferred to the complex TLR4-MD2 by LBP and CD14 allowing the recruitment of TIRAP in lipid rafts and signaling via Myd88 with early activation of NF-kB. The internalization of the complex causes the separation of TIRAP-Myd88 and the binding of TRAM that recruits Trif: the way Trif-dependent ends in the production of interferons through IRF3 and allows late activation of NF-kB and AP-1

1.5 Oxidative and nitrosative stress in chronic obstructive pulmonary disease (COPD)

Oxidative stress is a particular type of chemical stress induced by the presence in a living organisms of an excess of reactive chemical species, generally due to oxygen (reactive oxygen species (ROS)), also in response to an increased production of ROS and/or a reduction in the efficiency of the physiological antioxidant defense systems, can be produced nitrogen reactive species (RNS).ROS/RNS are continuously produced in the cells; an example of this production is located in the mitochondrial respiratory chain.

Oxidative stress occurs when ROS are produced in excess of endogenous antioxidant defence mechanisms (Ryter SW, 2007). This results in oxidization of a variety of biological molecules, such as lipids, proteins and deoxyribonucleic acid (DNA). Consequently these can lead to cell dysfunction or death, damage to extracellular matrix, and inactivation of key anti-oxidant defences (or activation of proteinases) (American Thoracic Society, 2012).

Oxidative stress has also reported as a major determinant of bronchial and lung alterations in patients with COPD (Ricciardolo et al, 2005) and it is also an important mechanism of amplification of the disease(Rahman I.). The biological markers of oxidative stress (eg. Hydrogen peroxide, 8-isoprostane,nitric oxide and lipid peroxidation products) are increased in the exhaled air breath condensate, sputum, and systemic circulation of patients with COPD, as well as urine of smokers(American Thoracic Society, 2012).Oxidative stress further increased in exacerbations. Oxidants are following inhalation of cigarette smoke and other particulates, and released from activated inflammatory cells such as macrophages and neutrophils (MacNee W.). In patients with COPD may also be a reduction of endogenous antioxidants. In the lung oxidative stress has many negative consequences that include activation of inflammatory genes, inactivation of anti-proteases, stimulation of mucus secretion and increased plasma exudation.

Many of these effects are mediated by the nitrogen peroxide that is formed by the interaction between the superoxide anion and nitric oxide. In turn, the nitric oxide is generated by inducible nitric oxide synthase (iNOS) expressed in peripheral airways and lung parenchyma of patients with COPD. Oxidative stress may also explain the

reduction of the histone deacetylase activity in the lung tissue of patients with COPD, which may lead to enhanced expression of inflammatory genes and also a reduction in the anti-inflammatory glucocorticosteroids(Ito K. et al., 2005).

1.5.1 Alteration of protease-antiprotease balance

There is an imbalance in the lungs of COPD patients between proteases that destroy connective tissue components and antiproteases that protect them. The production of oxygen reactive species (ROS) from the inflammatory response in addition to those derived from cigarette smoke or other irritants result in antiproteases becoming vulnerable to oxidation (Spurzem JR and Rennard SI, 2005). Several proteases, derived from inflammatory cells and epithelial cells, are increased in patients with COPD. Therefore, a positive feedback loop is created in which inflammation induces these imbalances, and the imbalances promote more inflammation (Scanlon, 2004). The following figure 24 displays the disproportion between proteases and antiproteases. The destruction of elastin by proteases, a major component of lung parenchyma connective, is an important characteristic of emphysema and is likely to be irreversible.

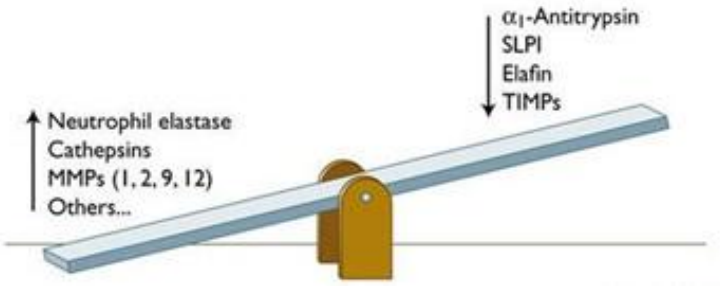


Fig. 24: Proteases and antiproteases involved in COPD

1.5.2 Redox species

There are different types of reactive molecules; these molecules can be radical or not radical (table 4). They can also be generated exogenously or produced by the cell starting from different sources.

Most common redox species are:

- Reactive oxygen species (ROS)
- Reactive nitrogen species (RNS)
- Carbon monoxide (CO)
- Hydrogen Sulfide (H2S)

ROS	RNS
▪ **Not-radicals**	▪ **Not-radicals**
Hydrogen peroxide (H2O2)	Dinitrogen tetroxide (N2O4)
Hypochlorous acid (HOCl)	Dinitrogen trioxide (N2O3)
Ozone (O3)	Cation nitric oxide (NO +)
Singlet oxygen	Nitric acid (HNO2)
Lipid peroxidase	Peroxynitrite (ONOO-)
	Nitrite (NO2-)
	Nitrate (NO3)
	S-nitrosothiols (RSNO)
▪ Radicals	▪ Radicals
Hydroperoxide (HO2 ·)	Nitric oxide (NO ·)
Hydroxyl (OH ·)	Nitric dioxide (NO2 ·)
Percossile (RO2 ·)	
Superoxide (O2 · -)	

Table 4: reactive oxygen and nitrogen species radicals and not radical

1.5.3 Oxygen Reactivity

The reactivity of molecular oxygen (O_2) and its partially reduced species can be explained by analyzing the structure of their molecular orbitals and the value of the potential reduction.

Oxygen is a di-radical stable with two unpaired electrons in the π antibonding orbitals (fig.25); one important consequence of this is that the organic molecules with unpaired electrons can transfer only one electron at a time to oxygen. Since oxygen is a relatively weak electron acceptor and many organic molecules are weak electron donors, oxygen is not able to oxidize in an efficient manner the amino acids and nucleic acids, however, it reacts readily with the unpaired electrons of transition metals and organic radicals. In contrast, the potential reduction of O_2•$^-$, H_2O_2 and OH• show how they are much more strong oxidizing then O_2 (fig.26).

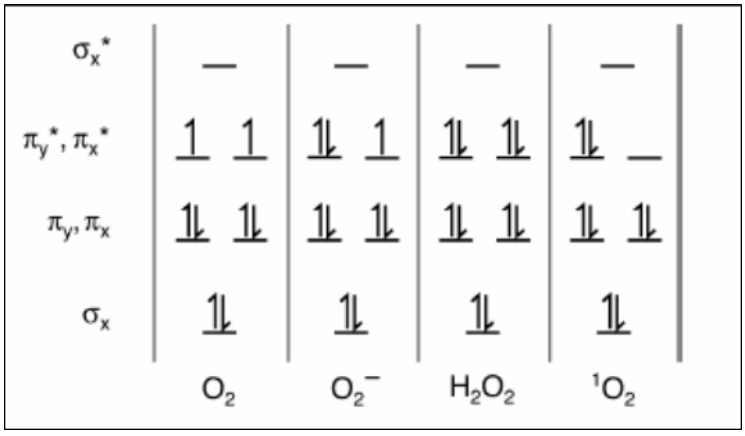


Fig.25: Diagram of the molecular orbitals of the oxygen (O_2), the radical superoxide (O_2• $^-$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$)

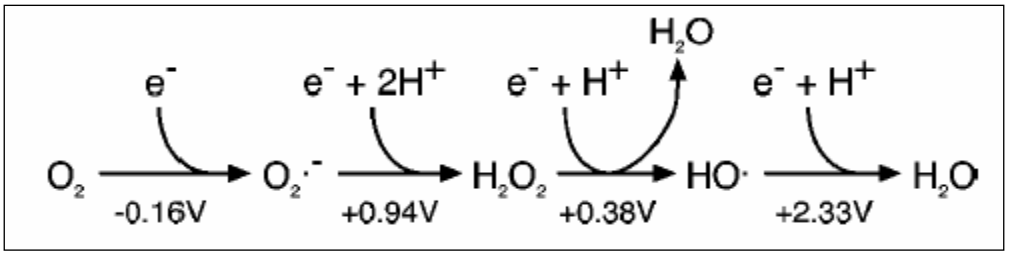


Fig.26: Potential reduction of molecular oxygen and its species

1.5.4 ROS Formation

ROS are generally produced during normal cellular metabolism, but their formation may be increased by particular conditions of the external environment such as exposure to ionizing radiation, the increase of the oxygen pressure or the presence of oxidants agents.

The toxicity of $O_2•^-$ depends mainly from the interaction of the latter with the H_2O_2. In fact, according to the Haber-Weiss reaction (Haber F & J. Weiss, 1934) the interaction of $O_2•^-$and H_2O_2can lead to the formation of OH•, the most reactive species partially reduced by oxygen:

$$O_2\cdot^- + H_2O_2 \rightarrow OH^- + HO\cdot + O_2$$

It has been shown that the constant of this reaction in water solution is close to zero (Richmond et al., 1981) and it cannot occur at low concentrations of $O_2•^-$and H_2O_2present in vivo. However, the in vivo presence of Fe^{+2} can catalyze the formation of OH•. In the presence of an electron donor, the Fe_3 + is reduced to Fe_2 + (1) and the oxidation of this ion by oxygen leads to the formation of $O_2•^-$ (2); the latter, following a dismutation reaction, form H_2O_2 (3) which can react with Fe_2 + by the Fenton reaction to form OH • (4)

1) $\text{Donatore}^{rid} + Fe^{3+} \longrightarrow \text{Donatore}^{ox} + Fe^{2+}$
2) $Fe^{2+} + O_2 \rightleftharpoons Fe^{3+} + O_2^{\cdot-}$
3) $O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$
4) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO\cdot + HO^-$

The Fe^{2+} may be produced, as had been shown by preliminary studies (Imlay JA, 2003), by the reverse reaction shown in equation (2). However this reaction in vivo has little importance because of the low concentration of $O_2•^-$(~ 10-10 M) (Imlay JA, Fridovich I. 1991). The reduction of Fe^{3+} in vivo, as demonstrated by Rowley and Halliwell (1982), could be due to the action of reducing agents such as the intracellular NAD(P)H and glutathione that are much more abundant although less reactive than $O_2•^-$ (Winterbourn CC, 1979). Recent studies have shown that both the FADH2 and the

cysteine residues can quickly reduce the Fe^{3+} in vitro (Woodmansee AN, Imlay JA, 2002; Park and Imlay, 2003), in addition treatments that increase their levels in vivo, increase the damage done by the formation of OH• also an order of magnitude.

As shown by equation (3), $O_2•^-$, following a dismutation reaction, form O_2 and H_2O_2. The latter is able to oxidize some organic compounds such as cysteine and methionine but oxidation occurs in vivo only when there are high concentrations of this compound. Therefore, the H_2O_2toxicity depends on its ability to generate •OH in the presence of $O_2•^-$ and Fe^{2+}.

1.5.5 ROS production inside the cell

ROS are generated during many cellular activities (fig. 27). Definitely one of the in vivo mechanisms that generates $O_2•^-$ and H_2O_2 is the loss of electrons by the transport respiratory chain. The site most likely involved in the production of these species is the bc1 complex in which there are components which easily undergo autoxidation as the cytochrome B566 and ubiquinone.

$O_2•^-$ and H_2O_2 are also formed by autoxidation of different flavoproteins such as NADH dehydrogenase, succinate dehydrogenase, the xanthina oxidase. Even the oxidation of small molecules such as flavins, catecholamines and tetrahydrofolate can lead to the formation of these reactive oxygen species.

In addition it has also been evidenced the production of $O_2•^-$ and H_2O_2 in cells that activate phagocytosis such as neutrophils, monocytes, macrophages and eosinophils when in contact with a foreign body or with the immuno-complex.

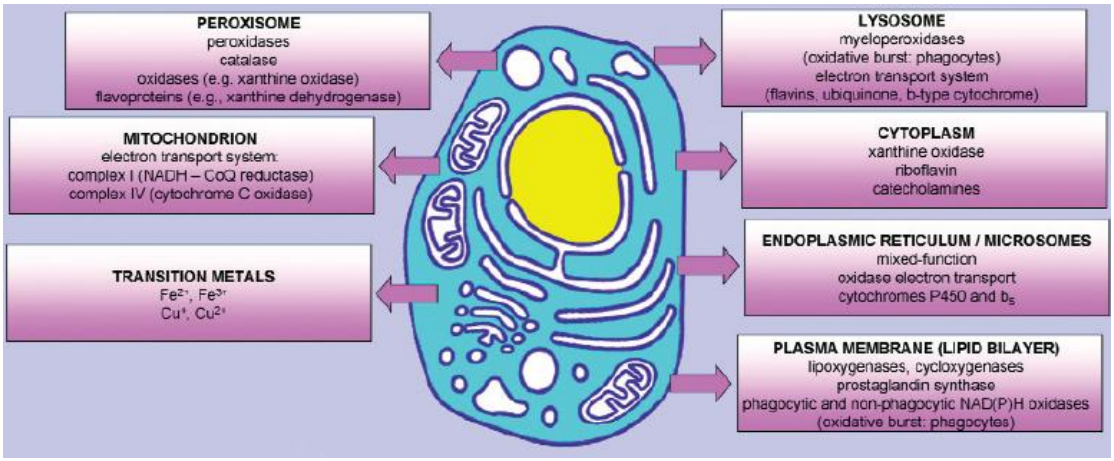


Fig. 27: Cellular resource of reactive oxygen species

1.5.6 Damage caused by ROS

The greatest damage procured by $O_2^{\bullet-}$ is directed to some classes of proteins. It has been shown that the radical is capable to inactivate enzymes which contain a cluster Iron-Sulfur (4Fe-4S) exposed to solvent such as aconitase B (Gardner PR and Fridovich I, 1991), the fumarase A and B (Liochev SI and Fridovich I. 1993) and the E. coli dihydroxy-acid dehydratase (Kuo CF. et al., 1987). The 4Fe-4S cluster is formed by three atoms of Fe coordinated by 4 bonds with sulfur and a quarter Fe atom coordinated to 3 bonds with the sulfur and responsible for the catalysis. The catalytic iron that has a positive charge attracts electrostatically $O_2^{\bullet-}$ and this interaction determines the oxidation of the cluster and the release of the iron atom (fig. 28). Iron-sulfur clusters of respiratory enzymes are damaged instead by $O_2^{\bullet-}$ because they are protected by the protein polypeptide chain and therefore are not exposed to the solvent.

Even the H_2O_2 can create direct damage to some biomolecules although the activation energy for the breaking of the O-O bond is high and limits its reactivity. H_2O_2 oxidizes the proteins cysteine residues generating sulfenic acids which can form crosslinks with other cysteines or be further oxidized to sulfinic acids. H_2O_2 can also oxidize methionine residues although this reaction is made at a still lower efficiency than the cysteine oxidation. In addition, H_2O_2 is able to directly oxidize iron-sulfur clusters of proteins. The interaction of H_2O_2 with the *d* orbitals of transition metals weakens the O-O bond causing the oxidation of the iron in the cluster.

As noted above, the species that directly causes the most damage to biomolecules is $OH^{\bullet}$ and most of toxicity H_2O_2 and $O_2^{\bullet-}$ is precisely determined by their ability to generate $^{\bullet}OH$. The damage caused in the cells by this radical are many because it reacts with almost all kinds of organic molecules present in living cells.The reactivity of $OH^{\bullet}$ is so high that it reacts immediately with any biological molecule in its vicinity producing secondary radicals with variable reactivity. In addition to oxidation and carbonylation of the amino acid residues and the peroxidation of lipids membrane, one of the most important damage that this radical induces in the cells is due to its interaction with DNA. $OH^{\bullet}$ is able both to subtract electrons to the nitrogenous bases and the deoxyribose and to add electrons to the basics with the consequent production of radicals that can determine different types of damage, such as rupture of the filaments or the mutations generation.

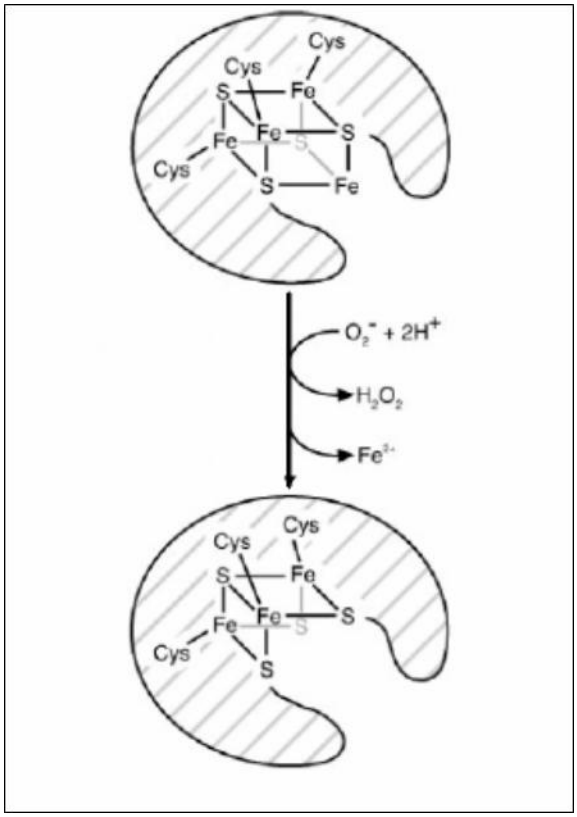


Fig. 28: Oxidation of Iron-Sulfur Cluster from $O_2^{\bullet-}$

1.6 Human Chaperonins

1.6.1 Heat shock proteins: A short overview

The term ***heat shock response*** was introduced for the first time in 1962 by the Italian scientist Ferruccio Ritossa. He discovered chromosome puffsafter the exposure to high temperatures of Drosophila salivarygland. In 1974 the products of these genes were isolated and subsequently called heat shock proteins (HSPs)(Tissières A. et al. 1974). Today Adrienne L. Edkins and Aileen Boshoff (2014)define HSPs as the guardians of protein homeostasis.

HSPsare among the most evolutionarily conserved proteins. This is documented by their high degree of conservation in different species (from bacteria to humans)and by their enormous abundance in all cells, playing similar roles in all the organisms (Csermely; 2001).Under physiological conditions, HSPs can account for approximately 1–2%of total cellular proteins and this can increase up to 4–6% after stress conditions (Garrido et al. 2001).

Heat shock protein are groupedinto severalfamiliesbased on their sizes from 7 kDa to 110 kDa: small Hsps, Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100 (Lindquist and Greig; 1988) and have been found in virtually every part of the cell, including nucleus, cytoplasm, and mitochondria (De Maio A. et al., 1995 and 1999; Wheeler DS et al., 2006 and 2007). In 2009, new guidelines for the nomenclature of the human HSPs families were proposed. In this classification, the human heat shock proteins have been renamed to the following: HSPH (former name HSP110), HSPC (HSP90), HSPA (HSP70), HSPD/E (HSP60/HSP10) and CCT (TRiC), DNAJ (HSP40), and HSPB (small HSP or sHSP) (Kampinga HH et al. 2009).(table 5). It has to be borne in mind that not all HSPs are chaperones and that not all chaperones are HSPs. The term chaperonin is applied to the chaperones with a molecular weight in the range 55-64 kDa and that have been classified into Group I and II. The members of each group are evolutionarily related: those of Group I are present in bacteria (eg. HSP60 is also named GroEL, or Cpn60) and in the mitochondria (eg. HSP60, also named Cpn60) of eukaryotes, those of Group II occur in the cytoplasm of archaea and in the cytosol of eukaryotic cell (CCT subunits) (Macario AJL et al., 2013).

Name	Size (kDa)	Localization	Bacterial Homolog	Some Known and Possible Functions
Ubiquitin	8	Cytosol/nucleus	—	Nonlysosomal degradation pathways
HSP 27 (HSPB1)	27	Cytosol/nucleus	—	Regulator of actin cytoskeleton; molecular chaperone; cytoprotection
Heme oxygenase	32	Bound to ER, extends to cytoplasm	—	Degradation of heme to bilirubin; resistance to oxidant stress
HSP 47	47	ER	—	Collagen chaperone
HSP 60 (HSPD)	60	Mitochondria	Gro EL	Molecular chaperone
HSP 70 (HSPA)	72	Cytosol/nucleus	Dna K	Highly stress inducible; involved in cytoprotection against diverse agents
	73	Cytosol/nucleus	—	Constitutively expressed chaperone
HSP 90 (HSPC)	90	Cytosol/nucleus	htpG	Regulation of steroid hormone activity
HSP 110	110	Nucleolus/cytosol	Clp family	Protects nucleoli from stress

Table 5. Major Heat Shock Protein Families. John S. Giuliano Jr. et al. 2011. The Open Inflammation Journal.

A particularly interesting aspect is that among the different member of one HSP family (but not among different families) stress proteins are highly homologous in nearly all species. Members of the HSP70 family share more than 50% of their amino acids sequences between different evolutionarily organisms as prokaryotic and eukaryotic (Lindquist S. and Craig E. A., 1988).

HSPs are an important class of proteins that have different functions essential for cell life and survival. Theyinhabit nearly all cellular compartments where they fulfill a broad variety of chaperoning functions include folding of nascent polypeptides and re-folding, regulation of protein import and export, assembly and disassembly of macromolecular structures, and support of antigen processing and presentation (Pierce; 1994, Hartl; 1996, Hartl and Hartl-Hayer; 2002; Macario AJL, Conway de Macario E, 2005). For their multiple functions and their mode of induction, they are also called molecular chaperones and stress protein.

In response to environmental stress, to avoid lethal cells damage induced by misfolding and protein aggregation, the synthesis of HSPs is highly up-regulated, while the synthesis of other proteins in general is down-regulated. Many stressful stimuli involved in this cellular response are: heat (as classical inducer), oxygen radicals, heavy metals, amino acid analogue (Hightower 1980) UV, gamma rays (Gehrmann M et al, 2005), cytostatics drugs (Gehrmann M et al, 2002;Ciocca DR et al, 2003), anti-

inflammatory drugs (Gehrmann et al, 2004), deprivation of nutrients, malignant transformation, bacterial and viral infections (Fuller et al, 1994).Furthermorea significant increase in cytosolic HSP is also detectable during cell replication and differentiation (Milarski et al, 1989).

During evolution, this class of proteins has also acquired "extra-chaperoning" roles, such as participating in immune system regulation amplifying the immune response by modulating antigen processing and/or by acting as autoantigens (Macario AJL et al. 2010), cell senescence (Macario AJL et al. 2010), cell differentiation, programmed cell death and carcinogenesis (Kottke T. et al, 2007). These molecules have also been implicated in the pathogenesis of a number of chronic inflammatory and autoimmune diseases, like inflammatory bowel disease (IBD), in which HSPs have been identified as potential biomarkers for diagnostics, prognostics and etiopathogenetic factors, or therapeutic targets (Rodolico V. et al. 2010; Tomasello G, 2011).

In respect to inflammatory lung diseases has been reported for the first time in 1995an increased expression of HSPs in bronchial epithelia cells (Vignola AM et al. 1995). This study demonstrated that the overexpression of HSPs in mild asthma may play an important role in protecting cells against injuries as well as in promoting and/or facilitating repair processes within the airways. However, these data are not in agreement with Fajac I. et al (1997) which showed an overexpression of HSPs (HSP60, HSP70 and HSP90) in both asthmatic patients and control subjects, not confirming that HSPs play an important role in inflammatory and immune responses in mild asthma.

Previous studies of our team showed increased levels of heat shock proteins (Hsp10, Hsp40 and Hsp60) in bronchial biopsies of COPD patients compared to Control Non-Smokers, suggesting an important role in the inflammatory response (Cappello et al, 2011). In addition, Hsps have other important functions in various metabolic mechanisms of neoplastic cells such as tissue invasion, induction of angiogenesis and metastasis (Cappello et al, 2005; Cappello F. and Di Stefano A., 2005).

1.6.2 Extracellular HSPs

Heat shock proteins are essential intracellular molecular chaperones (Lindquist S. and Craig, 1988), lacking transmembrane domains, that can be induced by a wide panel of stress signals and have strong protective properties in the cytosol(Gallucci and

Matzinger, 2001). In addition to the intracellular response, stress also triggers the release of proteins into the extracellular spaces (Mambula S.S. and Calderwood., 2006).HSPs such as HSP27, HSP60, HSP70, and HSP90 can also be found in the extracellular environment, or on the plasma membrane, where they play key roles in the stimulation of the immune system, interacting also with adjacent cells(Schmitt et al., 2007).The first reports of stress protein release involved Hsp70 secretion released from neuronal cells (Hightower L.E. and Guidon P.T.,1989). Now we know avariety of cell types that secrete stress proteins, including neuronal cells, monocytes, macrophages, B cells, and tumor cells of epithelial origin (Robinson M.B. et al., 2005; Clayton A. et al. 2005; Davies E.L. et al. 2006). In humans, their presence in the serum is associated with stress conditions, including inflammation, bacterial, and viral infections (Campisi J. And Fleshner M., 2003). In vitro, members of the HSP70 and HSP90 families have been detected in the medium of antigen-presenting cells (APCs)(Barreto, A, 2003). In addition, members of the HSP60 families have been found in the 16-HBE (human bronchial epithelium) supernatants (Cappello F. et al, 2011).

Although the immunological role of HSPs in extracellular environment presents several studies, the mechanism of transport to the plasma membrane, the membrane anchorage and the exportremains to be determined. HSPs lack the consensus signal for secretion via the classical Golgi pathway. The first hypothesis that was assumed that extracellular HSP originated on dead cells undergoing lysis (Basu et al., 2000). Different pathways have also been proposed for HSPs release: a lysosome–endosome pathway (Mambula and Calderwood, 2006) or a release by secretory-like granules (Evdonin et al., 2006). Another hypothesis supports that cytosolic HSPs are transported to the plasma membrane in concert with other proteins possessing transmembrane domains that fulfill shuttle functions and there is the possibility for membrane anchorage might be a direct interaction of HSPs with lipid components. De Maio et al. showed an association of members of the HSP70 family with phosphatidylserine (PS) in PC12 tumor cells (De Maio, 2011). It can be hypothesized that after binding of HSP70 to PS, a flip-flop mechanism might facilitate the transport of HSP70 from inside the cell to the outer membrane leaflet. Thus, several mechanisms have been proposed to account for the release of these HSPs into bio-fluids, however the question remains unresolved (Didelot et al., 2007; Joly et al., 2010; De Maio, 2011).

However, recent studies of our research groupstrongly suggest that human HSP60, the product of the HSPD1 gene, is released in extracellular environment by an

active secretion mechanism not due to cell damage or death with membrane disruption, but probably reflecting a general physiological phenomenon.Merendino AM and Cappello F (2010) showed for the first time, that human tumor cells can secrete HSP60 via exosomes, extracellular vesicles, released from normal and tumor cells by multivesicular bodies (MVB), with important roles in immune system activation

(Stoorvogel W, 2002; Johnstone RM, 2006).Campanella C. and Cappello F. in the 2012 also demonstrated that HSP60 localizes in the tumor cell plasma membrane, is associated with lipid rafts and integrated in the exosomal membrane. In this work, they also collected evidence in favor of a participation of the Golgi apparatus in HSP60 release from tumor cells. These data suggest that HSP60, but not HSP70, secretion from tumor cells involves the Golgi apparatus and that Golgi could be responsible of HSP60 import into exosomal vesicles.

Extremely interesting aspect but not still entirely clear is the behavior that HSPs have when released into the extracellular environment. When extracellular HSPs are released from damaged or stressed cells appear to act as local "danger signals" that activate stress (Giuliano JS Jr, 2011) inducing signal transduction cascades after binding surface receptors of adjacent cells (Calderwood SK, 2007). Many articles report that this extracellular stress proteins have powerful effects on the host innate and adaptive immune response(Srivastava PK, 2000 and 2001; Calderwood et al., 2005 and 2007; Giuliano JS Jr, 2011). HSPs in fact interact with the immune response in a number of different contexts. Mammalian cell, for example, express endogenous stress proteins to high levels after trauma, exposure to bacteria or bacteria proteins, virus or oxidative stress (Hunter-lavin C. et al., 2004).

As explained in the previous paragraphs, external or exogenous danger signals have traditionally been called pathogen-associated molecular patterns (PAMPs) and include pathogen-derived proteins, nucleic acids, and lipids such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid, CpG DNA, and flagellin. These PAMPs are recognized by a surprisingly limited number of highly conserved pattern recognition receptors (PRRs), which include the Toll-like receptors (TLRs), the nucleotide-binding oligomerization domain (NOD) receptors, C-type lectin receptors, NOD-like receptors (NLRs), RIG-I-like receptors, and the receptor for advanced glycation end-products (RAGE). However, it is now known that same PRR appear to recognize endogenous danger signals as well (Mollen KP, 2006;Giuliano JS Jr, 2011; Pouwels SD et al., 2014) hence the term, danger-associated molecular pattern or DAMP (Bianchi ME, 2007).

With this in mind, extracellular HSPs are commonly included in a growing list of a family of proteins known as danger-associated molecular patterns (DAMPs) or alarmins, which trigger an immune response to tissue injury, such as may occur with trauma, ischemia-reperfusion injury, oxidative stress, etc (Bianchi ME, 2007;Wheeler DS, 2007; Calderwood SK et al., 2007; Williams JH et al. 2008). In addition to the release of DAMPs, cell damage or death also induces the release of several cytokines and chemokines that can induce or regulate immune responses (Hirsiger, S., et al., 2012; Krysko, D.V. et al., 2012). An important characteristic of DAMPs is that they specifically bind PRRs,which upon ligation lead to activation of the innate immune system. This ligation activate downstream signaling pathwaysincluding nuclear factor-kB (NF-kB), mitogen-activated protein kinase, and type I interferon pathways, initiating the release of pro-inflammatory cytokines and chemokines (e.g., IL-6, IL-8, type I interferon, and tumor necrosis factor), and ultimately resulting in activation of the immune system and attraction of immune cells to the site of damage(Chen, G.Y. et al., 2010) (fig. 29).

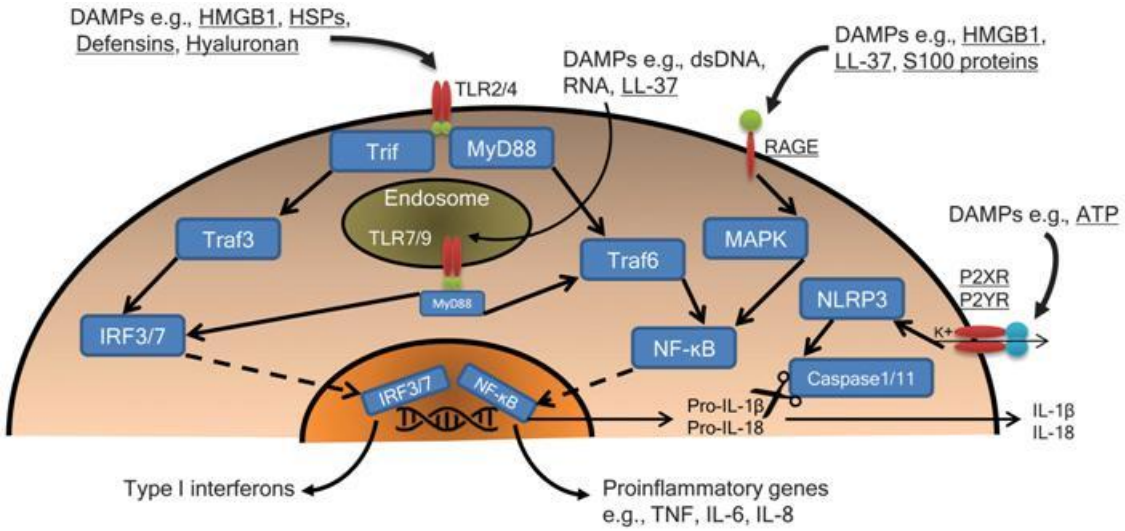


Fig.29: Ligation of pattern recognition receptors (PRRs) by damage-associated molecular patterns (DAMPs), relevant for chronic obstructivepulmonary disease (COPD), initiates the release of pro-inflammatory cytokines by multiple pathways.Modified from Pouwels SD et al. 2014. Mucosal Immunol. 7(2):215-26.

HSPs have been shown to activate both TLR2 and TLR4 signaling in a MyD88-dependent(MyD88/TRAF6/NF-kB) pathway with the translocation of NF-kB in the nucleus where it induces transcription of pro-inflammatory genes, including tumor

necrosis factor (TNF), interleukin (IL) -6 and IL-8 (Joly, A. et al., 2010; Tolle L.B., and Standiford, T.J., 2013),or with the activation of Trif/TRAF3/IRF3 signaling pathway and subsequent nucleus transcription of type I interferons after IRF3translocation. Hence the term ***chaperokine,*** introduced for the first time to describe the function of extracellular HSPs (Asea, A, 2000).

In recent years, several studies have been conducted to investigate the role of HSPs in COPD through "in vivo" studies or adopting "in vitro" models. The first studies conducted in the nineties using "in vitro" models, have demonstrated an increased levels of HSP70 and HSP90 in the supernatants of on monocytes exposed to cigarette smoke (CS) compared to not exposed cells (Pinot, F., 1997). More recently, Hacker, S. et al. 2009 have shown significantly higher levels of HSP27, HSP70 and HSP90 by analyzing serum of COPD patients compared with control nonsmoking subjects. Furthermore, the authors have found increased levels of HSP10, HSP27, and HSP40 protein in airway epithelial cells of COPD patients in comparison with healthy controls and control smokers (Hacker, S. et al. 2009). These data confirm the data obtained by our research group, where increased HSP10, HSP40 and HSP60 expression have been observed in bronchial biopsies of patients with severe COPD (GOLD stage III/IV) compared with healthy nonsmoking volunteers (Cappello F. et al. 2011).This increase in HSP60 was positively correlated with neutrophil numbers in the biopsies, an important pathological hallmark of COPD.However, "in vitro" experiments on human bronchial epithelial cells (16HBE) actively released HSP60 upon H_2O_2 stimulation, to mimic oxidative stress in COPD (Cappello F. et al. 2011). Further experiments on umbilical cord cells exposed to CS have increased levels of HSP60 released (Kreutmayer, S.B. et al., 2011), suggesting that cigarette smoking plays a key role in the HSP60 secretion. An increased expression and release of several HSPs has been found in the circulation and lungs of COPD patients. Although some studies only examined intracellular expression of HSPs, increased expression may cause increased release upon accidental necrosis. The role of HSPs in COPD is not fully elucidated, for this reason further studies are needed to determine whether HSPs may play a causal role in airway inflammation and pathogenesis of COPD.

In the next section will be explained the specific action mechanism of extracellular HSP60 and the cascade of events that are triggered by the interaction with TLRs, trying to show the anti- or pro-inflammatory behavior of this molecule, still little known.

1.6.3 Heat Shock Proteins-60 (HSP60)

Heat Shock Proteins-60 (HSP60)was one of the first chaperones studied. Initially called chaperonin, it is a 60 kilodalton oligomer composed of a monomers complex formed of two stacked heptameric rings (Cheng MY, 1990). Like all heat shock proteins, also HSP60 is a evolutionarily conserved protein. The significant function, structural, and sequential homology between HSP60 and its prokaryotic homolog, groEL, demonstrates this level of conservation (Macario AJL, et al.,2013).HSP60 plays an essential role in protein folding, refolding, trafficking and degradation but also in the regulation of cell growth and differentiation, apoptosis and cell-to-cell crosstalk, inflammation, and tissue repair (Macario AJL et al, 2005; Calderwood et al, 2007). In addition, studies have shown that HSP60 plays an important role in replication of mitochondrial DNA and in the transport and maintenance of mitochondrial proteins.

The folding and refolding ability is due to double ring structure that forms a large central cavity in which the unfolded protein binds via hydrophobic interactions(Fenton WA, et al., 1994). EachHSP60subunithas has three domains: apical, equatorial and intermediate domain(Ranford JC,et al., 2000). In equatorial domain is present the binding site for ATP and heptameric ring. The intermediate domain has the function to induce a conformational change when bound ATP. This allows the alternation between the binding sites of the hydrophilic and hydrophobic substrate. Furthermore the intermediate domain serves to bind the equatorial and apical domain together(Ranford JC,et al., 2000). HSP60 when is in a hydrophobic state is inactivated. However, when activated by ATP, the intermediate domain undergoes a conformational change that exposes the hydrophilic region. This insures that the protein binding is formed. Chaperonin 10 (HSP10/Cpn) forms a complex with HSP60 helping it in folding acting, closing HSP60 in the upper end. This causes the central cavity to enlarge and aids protein folding (Ranford JC, et al., 2000; Deocaris CC, 2006).Another important aspect was demonstrated by Itoh H et al.: in addition to its typical location in the mitochondria, HSP60 can also be found in the cytoplasm under normal physiological conditions(Itoh H et al, 2002).

it is now known that HSP60 plays a key role in both intracellular and extracellular environment. Many research studies have identified HSP60 incytoplasm, associating its involvement in several diseases. For example it is reported that HSP60 is

associated with different cancers. Cancer cells in fact express high levels of HSP60 compared to normal cells, and need them for survival (Seigneuric et al., 2010). Recent publications confirmed HSP60 as a possible biomarker. Clinical data from patients with localized and locally advanced prostate cancer showed an association between IHC expression of HSP60 and tumor progression. A study performed in human prostate cancers indicates that HSP60 expression assessed via IHC increases in both early and advanced prostate cancer when compared with non-neoplastic prostatic epithelium (Cornford et al., 2000). It was shown that, for patients with intense HSP60 staining in biopsy, recurrence-free survival was shorter than in those with weak expression (Glaessgen et al., 2008). The prognostic significance of HSP60 in cervical cancer suggest that HSP60 may be involved in the development of the disease (Hwang et al., 2009). HSP60 mRNA levels were significantly higher in primary breast cancer compared to healthy breast tissues. Using IHC, it was found that HSP60 expression increased from normal to invasive tissues, with an increased expression of HSP60 in tumors of advanced clinical stage when compared with earlier stage carcinomas(Isidoro et al., 2005; Desmetz et al., 2008). HSP60 was also identified by antibodies in sera from patients with chronic hepatitis, liver cirrhosis, or hepatocellular carcinoma (Looi et al., 2008). In colorectal carcinoma, the elevated expression of HSP60 showed a significant association with tumor differentiation, and may indicate a worse prognosis (Mori et al., 2005).

On the other hand is not yet clear the extracellular human HSP60 (HSPD behavior. It appears to be involved in many autoimmune and inflammatory processes within the body (Jamin C. et al., 2005). For example, circulating antibodies to human HSP60 (HSPD) appear to play a role in the pathophysiology of a number of vasculopathies and related illnesses. Elevated IgG antibodies to HSO60 (HSPD) are found in the sera of patients with rheumatic autoimmune diseases (Yokota SI et al., 2000), carotid artery atherosclerosis (Xu Q et al., 1993), borderline hypertension (Frostegard J. et al., 1997) and coronary artery disease (Hoppichler F et al., 1996). Xu and colleagues discovered an association between elevated circulating levels of HSP60 (HSPD) and the progression and severity of carotid artery atherosclerosis (Xu Q. et al. 2000). Patients with a sustained elevation of serum HSP60 (HSPD) were found to be at risk for the development of early atherosclerosis (Xiao Q., et al., 2005). Another confirmation comes froman animal model of atherosclerosis, where auto-antibodies to HSP60 were shown to be involved in disease progression (Foteinos G. et al., 2005).

It is becoming clearer that extracellular Hsp60 (HSPD) plays a role in inflammation and the body's immune response. In recent years has been demonstrated that HSP60 is able to bind to TLRs present on both innate immune cells (macrophages and dendritic cells) (Chen W. et al, 1999, Ohashi, K. et al., 2000, Habich, C. et al., 2002) and adaptive immune cells (T cells and B cells) (Zanin-Zhorov A. et al. 2003, Cohen-Sfady M. et al., 2005). HSP60 appears to act primarily via the TLR4 pathway (Vabulas RM et al., 2001; Ohashi K et al., 2000), but it has also been observed the activation of the TLR2 pathway (Vabulas RM et al., 2001). An extremely interesting aspect due to the fact that TLR signaling by HSP60 can induce responses that are either pro-inflammatory or anti-inflammatory depending on the cell type and the concentration of HSP60. T cells, for example, with a concentrations of nanogram per milliliter of HSP60 respond via TLR2 (Zanin-Zhorov A. et al. 2003 and 2006, Zanin-Zhorov A. et al., 2005); macrophages, B cells, and dendritic cells respond via TLR4 to microgram concentrations of HSP60 (Flohe, S. . et al., 2003; Habich, C et al., 2003). Macrophages and dendritic cells respond to HSP60 by activation and secretion of Th1 inflammatory cytokines such as IL-12, TNFα and interferon (IFN)-γ (Chen W. et al, 1999; Flohe, S. . et al., 2003). This aspect suggests a pro-inflammatory behavior.

However, HSP60 also appears to activate the adaptive immune response. The innate effect of HSP60 on B cells and T cells shifts their phenotype toward an anti-inflammatory response: HSP60 down-regulates T cell chemotaxis (Zanin-Zhorov A. et al. 2003) and secretion of IFN-γ and TNF-α (Zanin-Zhorov A. et al., 2005). HSP60 through activation of TLR2 pathway increases the secretion of IL-10 by T cells (Zanin-Zhorov A. et al. 2006). Additionally, HSP60 induces B cells to proliferate and secrete IL-10 through a signaling pathway that required TLR4 and MyD88, but not TLR2 (Cohen-Sfady M. et al., 2005).

In the end it is an oversimplification to think that HSP60 is a “danger signal” only (Chen W. et al, 1999). HSP60 can modulate the immune response in various ways. However, the function, mechanism and the possible signal pathways involved after HSP60 stimulation in lung epithelial cells have not been well studied. Therefore an objective of this thesis is concerned to investigate the response of respiratory epithelium when stimulated with human extracellular HSP60.

2 Aim of the thesis

We performed the present research project to investigate and explore the activity and functions of human low endotoxin HSP60 on human bronchial epithelial cells used as a single stimulus or in relation to oxidative, inflammatory and bacterial challenges. The moleculare pathways following these challenges were also studied.For this reson we performed in vitro experiments to address if HSP60 has a prevalent pro- or anti-inflammatory effects in extracellular environment.

3 Materials and Methods

3.1 "Ex vivo" experiments

3.1.1 Subjects

All Subjects were recruited from the Section of Respiratory Medicine of the Fondazione Salvatore Maugeri (Veruno, Italy). Particulary, we examined bronchial biopsies from 55subjects by immunohistochemistry (table 6); 11 were never-smokers with normal lung function (healthy non-smokers); 12were current- or ex- smokers with normal lung function (healthy smokers), 32had a diagnosis of COPD, of with 14 were classified as mild/moderate COPD and 18as severe/very severe COPD. The severity of the airflow obstruction was staged using GOLD criteria (2001) (GOLD: Global Initiative for Chronic Obstructive Lung Disease, 2013). All former smokers had stopped smoking for at least one year. COPD and chronic bronchitis were respectively defined, according to international guidelines, as: the presence of post-bronchodilator forced expiratory volume in one second (FEV_1) / forced vital capacity (FVC) ratio <70% or the presence of cough and sputum production for al least 3 months in each of two consecutive year. All COPD patients were with no previous exacerbation in the 6 months before bronchoscopy. None of the subjects was treated with theophylline, antibiotics, antioxidants, mucolytics, and/or glucocorticoids in the month prior to the bronchial biopsy. The study conformed to the Declaration of Helsinki, Ethics Consent was obtained, bronchial biopsies were performed according to the local Ethics Committee Guidelines, and informed consent was obtained from each subjects.

3.1.2 Lung function tests and volumes

Pulmonary function tests were performed as already described (Di Stefano et al; 2009). Pulmonary function tests included measurements of FEV_1/FVC under baseline conditions in all the subjects examined (6200 Autobox Pulmonary Function Laboratory; Sensormedics Corp., Yoba Linda, CA).Predicted values for the different measures were those from the Communité Européenne du Carbon et de l'Acier. In order to assess the reversibility of airflow obstruction and postbronchodilaor functional values, the FEV_1 and FEV_1/FVC% measurements in the groups of subjects with FEV_1/FVC%≤70% pre-bronchodilator was repeated 20 min after the inhalation of 0.4 mg of salbutamol.

Table 6: Clinical characteristics of the patients studied with techniques Immunohistochemistry

	Control non Smokers	Control Smokers	COPD mild/moderate	COPD Severe/ very severe
Subjects	11	12	14	18
Age (years)	67 ± 1	61 ± 7	67 ± 8	66 ± 9
M/F	10/1	9/3	12/2	11/7
Pack/year	0	43 ± 26	40 ± 19	54 ± 36
Smoker/Ex Smoker	0	2/10	5/9	13/5
FEV_1 pre-β_2 (% predict)	116 ± 14	104 ± 13	66 ± 14*	35 ± 8*#
FEV_1 post-β_2 (% predict)	ND	ND	72 ± 12	38 ± 9
FEV_1/FVC (%)	85 ± 10	81 ± 6	60 ± 8*	44 ± 10*#
Chronic Bronchitis	0	5	8	6

Data are expressed as mean ± standard deviation.

** Significantly different from controls non-smokers, p <0.0001; # Significantly different from mild / moderate COPD, p <0.0001.*

FEV1: forced expiratory volume in one second

FVC: forced vital capacity

3.1.3 Fiberoptic bronchoscopy, collection and processing of bronchial biopsies

Subjects attended the bronchoscopy suite at 8.30 am after having fasted from midnight and were pre-treated with atropine (0.6 mg IV) and midazolam (5-10 mg IV). Oxygen (3 l/min) was administered via nasal prongs throughout the procedure and oxygen saturation was monitored with a digital oximeter. Using local anesthesia with lidocaine (4%) to the upper airways and larynx, a fiberoptic bronchoscope (Olympus BF10 Key-Med, Southend, UK) was passed through the oral passages into the trachea.

Further lidocaine (2%) was sprayed into the lower airways, and four bronchial biopsy speciemens were taken from segmental and subsegmental airways of the right lower and upper lobes using size 19 cupper forceps. Bronchial biopsies for immunohistochemistry and qRT-PCR were gently extracted from the forceps and processed for light microscopy. Two samples were embedded in Tissue Tek II OCT (Miles Scientific, Naperville, IL), frozen within 15 min in isopentane pre-cooled in liquid nitrogen, and stored at -80 °C. The best frozen sample was then oriented and 6 µm thick cryostat sections were cut for immunohistochemical light microscopy analysis and 30 µm thick cryostat section were cut for qRT-PCR and processed as described below. Bronchial biopsies for eventual western blot analysis were immediately placed on ice, frozen in liquid nitrogen and processed as described below (fig. 30).

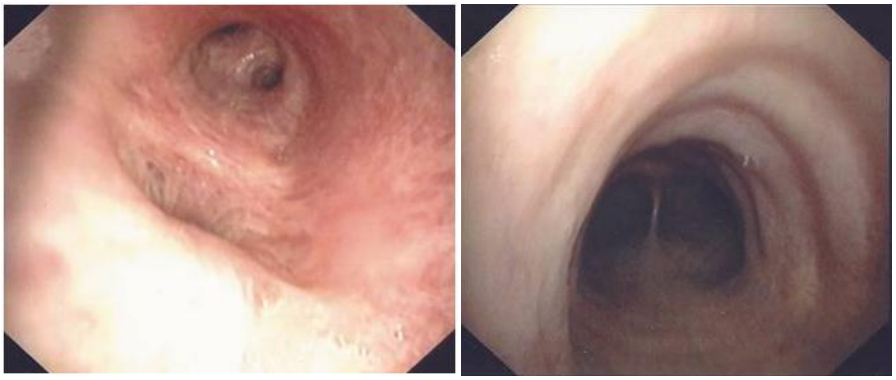


Fig. 30: Images captured during collection of bronchial biopsies during the bronchoscopy

3.1.4 Immunohistochemistry

Two section were stained with immunohistochemical methods. The best immunostained section was then selected for quantitative purposes. The following antibodies in table 7were used. Briefly, after blocking non-specific binding sites using serum derived from the same animal species as the secondary antibody, primary antibodies were applied at optimal dilutions in TRIS-buffered saline (0.15 M saline containing 0.05 M TRIS-hydrochloric acid at pH 7.6) and incubated (1h) at room temperature in a humidified chamber. Antibody binding was demonstrated with the use of secondary antibodies anti-mouse (Vector, BA 2000), anti-rabbit (Vector, BA 1000) or anti-goat (Vector BA 5000) (table 8) followed by Streptavidin AB Compex/AP, HRP (Dako, K0391) and fast-red or diamonobenzidine substrates.The nuclei were stained with hematoxylin. Control slides were included in each staining run using human tonsil or nasal polyp as a positive control for all immunostaining performed. For the negative

control slides, normal goat or rabbit non-specific immunoglobulins (Santa Cruz Biotechnology) were used at the same protein concentration as the primary antibody.

Antibody	Code	Specis	Concentration
TLR4	R&D System, AF1478	Goat	1:80
MYD88	S.Cruz, SC-11356	Rabbit	1:150
IL-8	R&D System, AF208-NA	Goat	1:50
IL-10	R&D System, AF217-NA	Goat	1:100
p65	S. Cruz, SC-372,	Rabbit	1:200
pp38MAPK	Epitomics; 1229-1	Rabbit	1:25
pERK1/pERK2	Epitomics; 1229-1	Rabbit	1:50
pSAPK/JNK	S.Cruz; SC-6254	Mouse	1:300
HSP60	S.Cruz; SC-1052	Goat	1:100

Table 7: code, species and concentration of use of primary antibodies used in immunohistochemistry

Antibody	Code	Concentration
Biotinylated anti-goat IgG	Vector, BA 5000	1:200
Biotinylated anti-mouse IgG	Vector, BA 2000	1:200
Biotinylated anti-rabbit IgG	Vector, BA 1000	1:200

Table 8: code and concentration of use of secondary antibodies used in immunohistochemistry

3.1.5 Scoring system for immunohistochemistry

Morphometric measeurements were performed with a light microscope (Leitz Biomed, Leica Cambridg,UK) connected to a video recorder linked to a computerized image system (Quantimet 500 Image Processing and Analysis System, Softwere Qwin

V0200B, Leica). Light-microscopic analysis was performed at a magnification of 630X. Immunostained cells were quantified in the area 100 µm beneath the epithelial basement membrane in several non-overlapping high power fields until all the available are was covered. The final result, expressed as the number of positive cells per square millimeter, was calculated as the average of all the cellular counts performed in each biopsy. We quantified the immunostained cells with at least a portion of the nucleus seen close to immunopositivity. The immunostaining for all the antigens studied was also scored (range: 0: absence of immunostaining, 1: from 1% until 33% of immunostained cells, 2: from 34% until 66% of immunostained cells, 3: from 67% to 100% immunostaining) in the intact (constituted by columnar and basal epithelial cells) bronchial epithelium, as previously described (Di Stefano et al., 1994). The final result was expressed as the average of all scored fields performed in each biopsy. A mean ± SD of 0.700 ± 0.260 millimeters of epithelium was analyzed in CODP patients and Control subjects.

3.2 In Vitro experiments

3.2.1 Cell cultures and treatments

We used the SV40 large T antigen–transformed 16-HBE (Human Bronchial Epithelial) cell line. HBE is a cell line that retains the differentiated morphology and function of normal human airway epithelia. For our experiments, 16-HBE cells were maintained in Dulbecco-modified Eagle's medium (DMEM) with 10% fetal bovin serum (FBS) and supplemented with 50 µg/ml penicillin, 50 µg/ml streptomycin, 1x non-essential amino acids, 1 mM sodium pyruvate and 2 mM glutamine. Cell culture were maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C. Passage number of cells used in this study ranged from 20 to 26. When cells were at 70–80% confluent, prior to each treatment, cells were starved with Dulbecco's modified Eagle's medium without FBS for 24 hours to make them quiescent. This ensured that all the cells were synchronised in the same phase of the cell cycle and that when stimulated their responses would be similar. The experiments were performed on 24-wells culture plates for mRNA and on T-25 flasks for Western Blotting experiments. The experiments started when the cells were at 60–70% confluent.

The 16-HBE were cultured for 1, 2, 4, 8 and 24 h in the presence and in the absence of LPS 10 µg/ml (Sigma-Aldrich, IT), H_2O_2 100 µM (Sigma-Aldrich, IT), Citomix (IL-1β 1 ng/mL, TNFα 10 ng/mL and INF-γ 10 ng/mL) and low endotoxin HSP60 1 µg/mL (Enzo Life Sciences, IT). Furthermore, the cells were treated combining HSP60(1µg/mL) with LPS (10 µg/ml), H_2O_2 (100 µM) and Citomix (IL-1β 1 ng/mL, TNFα 10 ng/mL and INF-γ 10 ng/mL). In some experiments, the p38 inhibitor SB203580 (Santa Cruz; 10µM, 30 min),was added to the 16HBE cultures before stimulation with LPS. At the end of stimulation, cell pellets and cell culture supernatans were collected for further evaluations. Cells were harvested immediately after incubation. Before mRNA isolation, cells culture were initially washed with phosphate buffer solution (PBS). All experiments were performed at least in quadruplicate.

3.2.2 16HBE RNA extraction and quantification

Total cellular RNA from exposed and non-exposed cultures was purified and isolated using RNAspin Mini RNA Isolation kit following manufacturer's instructions (GE Healthcare). Total RNA was resuspended in 100 µl nuclease-free water. RNA/DNA concentration was determined using a UV/visible spectrophotometer (λ260/280 nm, Eppendorf Bio Photometer plus) and stored at -80 °C.

3.2.3 Real-time RT-PCR

One-step real-time PCR was carried out by amplifying mRNA using the QuantiFast^{TM} SYBER Green RT-PCR Kit (Qiagen, IT) according to the manufacturer's instructions and the gene specific primers (Qiagen, IT)(table 9). Briefly, the PCR reaction mix, prepared in a total volume of 25 µl, was run on the Rotor Gene Q (Qiagen, IT) and the following PCR run protocol was used: 55 °C for 10 min (reverse transcription); 95 °C for 5 min (PCR initial activation step); 40 amplification cycles of 95 °C for 5 s (denaturation) and 60 °C for 10 s (combined annealing/extension), followed by melting curve analysis to ensure the specificity of PCR amplification. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was used as the reference gene for every target gene per sample, and the data were normalized against the respective GAPDH signaling. Cycle threshold (C_T) values were determined using the Rotor Gene

Q software (Rotor-Gene Q Series Software 2.0.2). Relative transcript expression values were obtained by normalizing C_T values of the target genes with C_T values of the house-keeping gene copy number.

Molecule	Primer Code
GAPDH (House-keeping)	QT01192646
IL-8	QT00000322
IL-10	QT00041685
p-38α (MapK 14)	QT00079345
JNK1 (MapK 8)	QT00091056
ERK1 (MapK 1)	QT00065933
p65	QT01007370
CREB 1	Ivitrogen

Table 9: list of primers used for qRT-PCR

3.2.4 Western blotting for p-p38 MAPK and IL-8 in 16-HBE after LPS, H2O2 and HSP60 stimulation

Whole cell proteins were extracted from Human Bronchial Epithelial cells (16-HBE) after LPS, H_2O_2 and HSP60 stimulation. In brief, frozen 16-HBE cell pellets were resuspended with mechanical disruption in RIPA lysis buffer with a protease inhibitor cocktail i frozen to –80°C and thawed after 24h. Particulate matter was removed by centrifugation at 12000 x g for 10 min at 4°C. Protein concentration was measured in the supernatant by the Bradford method according to the manufacturer's instructions (Bio-Rad Laboratories, Hemel Hempstead, UK). An equal volume of Laemmli sample buffer 2X concentrate was added to the final volume of the sample. At least 30 µg/lane of whole-cell proteins were subjected to a 12% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose filters (Hybond-ECL, Amersham Pharmacia Biotech) by blotting. Filters were blocked for 30 minutes at room temperature in Tris-buffered saline (TBS), 0.05% Tween 20, 3% BSA. The filters were then incubated with rabbit anti-human p-p38α (1229-1 Epitomics) or mouse anti-human IL-8 (I2519;Sigma-Aldrich) overnight at 4°C in TBS, 0.05% Tween 20, 1% BSA at

dilution of 1:000for anti-human p-p38α and at dilution of 1:500 for IL-8 . As positive controls 50 ng of human recombinant CXCL8 (IL-8) (Sc-4600; Santa-Cruz) was used. Then, filters were washed five times in TBS, 0.5% Tween 20 and then incubated for 1hat room temperature with ECL Peroxidase labeled anti-rabbit antibody conjugated (NA934VS,GE Healthcare, UK) in TBS, 0.05% Tween 20, 1% BSA, at a dilution of 1:10000 for p-p38α or with ECL Peroxidase labeled anti-mouse antibody conjugated (NA931VS,GE Healthcare, UK) in TBS, 0.05% Tween 20, 1% BSA, at a dilution of 1:3000 for CXCL8. After five further washes in TBS, 0.05% Tween 20 visualization of the immunocomplexes was performed using the ECL as recommended by the manufacturer (Amersham Pharmacia Biotech). As an internal control we incubated each filter with an anti-human β-actin antibody (A2228, Sigma-Aldrich). The 43kDa (β-actin) or 8kDa (CXCL8) and 38kDa (p-p38α) bands were quantified using densitometry with TotalLab TL120 (nonlinear Dynamics LTD, New Castle, UK) and expressed as a ratio with the corresponding actin optical density value of the same lane.

3.3 Data analysis

Group data were expressed as mean ± standard error for functional data or median (range) for morphologic data. We tested for a normal distribution for functional data (i. e. FEV1%, FVC, age etc.) ad for a non normal distribution for morphologic parameters. Then we applied the analysis of variance (ANOVA) in comparing subgroups of patients and control subjects for functional data. The non parametric Kruskal Wallis test was applied for multiple comparisons, without application of Bonferroni correlation, when morphologic data were analysed followed by the Mann-Whitney U test for comparison between groups. The statistical Guide to GraphPad Prisma recommends that the Bonferroni correlation should not be used when comparing more than 5 variables due to the conservative nature of the test and the subsequent likeliness of missing real differences. We believe that this comparative analysis is of value and represents part of our informative findings. For this reason we applied specific non parametric statistical tests to our data without including the Bonferroni correlation.

To verify the degree of association between functional or morphological parameters, in all smokers with and without COPD or in smokers with COPD alone the correlation coefficients between functional-morphological and morphological-morphological data were calculated using the Spearman rank method. Probability values of $p<0.05$ were considered significant. Data analysis was performed by using the Stat View SE Graphics program (Abacus Concepts Inc., Berkelay, CA).

In vitro data were expressed as mean±standard deviation and analysed by the t-test. Correlation coefficients were calculated using the Spearman rank method. Probability values of $p<0.05$ were considered significant. Data analysis was performed using the Stat View SE Graphics program (Abacus Concepts Inc., Berkeley, CA-USA).

4 Results

4.1 EX-VIVO

4.1.1 Clinical characteristics of subjects studied by immunohistochemistry

We obtained and studied bronchial biopsies from 55 subjects: 33 with stable COPD, 12 were current or ex-smokers with normal lung function, and 11 were non-smokers with normal lung function (table 6). COPD patients were divided into two groups: mild/moderate (GOLD stage I-II, n=14) and severe/very severe GOLD stage III-IV, n=18) [www.goldcopd.com]. Subjects in all four groups were age-matched. The smoking history was similar in the three smoking groups. Values of FEV1 (% predicted) and FEV1/FVC (%) were significantly different in the groups with mild/moderate and severe/very severe COPD compared to both control groups (healthy smokers and healthy non-smokers). Severe/very severe COPD patients also differed significantly from mild/moderate COPD patients (for overall groups, ANOVA test: $p<0.0001$ for FEV1% predicted and FEV1/FVC% values). Thirty-six percent (n=12) of the total COPD patients and 25% (n=4) of healthy smokers with normal lung function also had symptoms of chronic bronchitis. There was no significant difference when COPD patients and healthy smokers were compared for the presence of chronic bronchitis.

4.1.2 Immunohistochemical expression of TLRs and HSPs in the bronchial epithelium and lamina propria ofCOPD patients and control subjects

In the bronchial epithelium the score number (0-3) of HSP60 immunoreactive cells was significantly increased in Severe/Very severe COPDsubjects (median ± IQR; 1.5 ±0.4) compared with control non-smokers with normal lung function (median ± IQR; 0.7±0.3); Mann Whitney: p=0.006 (Fig. 1; panel f).No significant differences were observed between smokers with normal lung function and COPD patients(Fig. 1). Data are shown in table 10

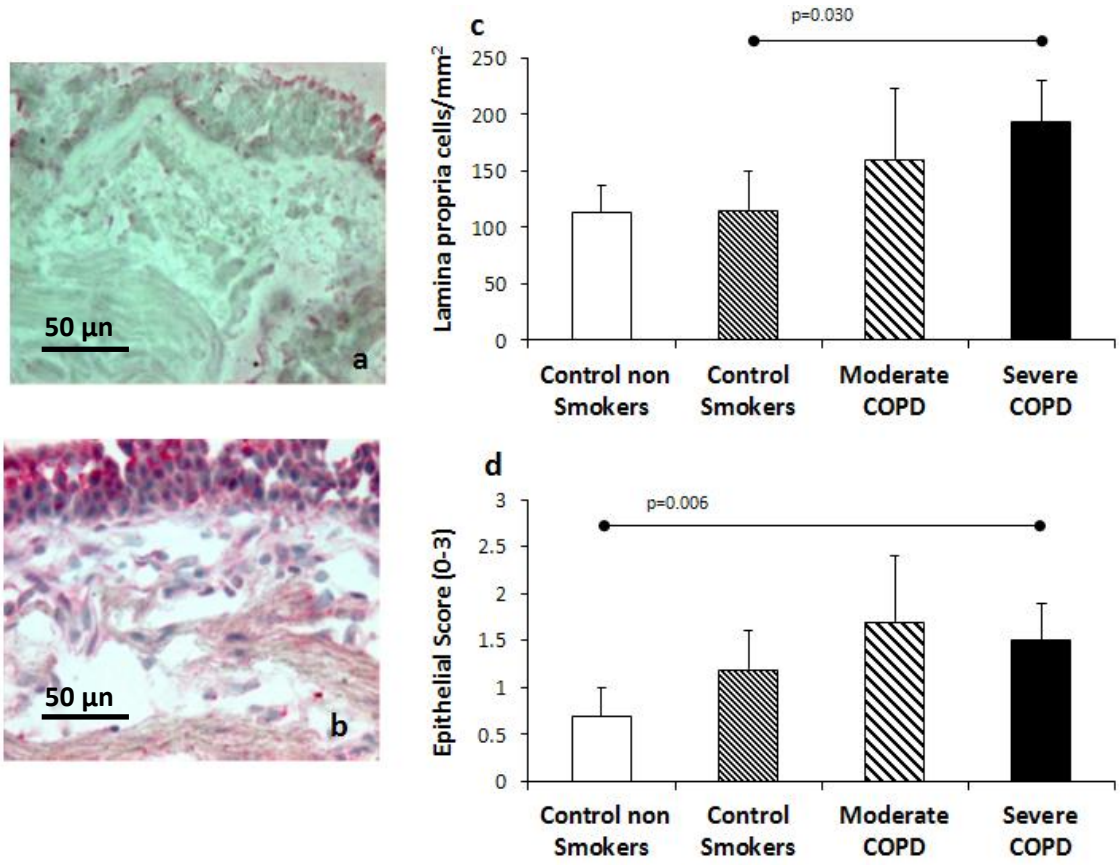


Fig 1: Photomicrographs showing the bronchial epithelium of a control non-smoker (panel a), and patient with chronic obstructive pulmonary disease (COPD) (panel b) immunostained for identification of HSP60+ cells. Results are representative of those from 9 nonsmokers, 16 healthy smokers and 33 subjects with stable COPD. Internal scale bars= 50 µm.HSP60 positive cells in the lamina propria (panel c) and epithelium (panel d) of subjects with chronic obstructive pulmonary disease (Moderate COPD; Severe COPD), control smokers and control non-smokers. The results are expressed as the median ± IQR positive cells per millimetre of epithelium. Statistical analysis: Mann-Whitney U-test for comparison between groups. Nonsmokers: n=9; healthy smokers: n=16; COPD: n=33. *: p<0.05.

In the lamina propria the total number of HSP60 positive cells (cells/mm^2) was not statistically different between control non-smokers and COPD patients. However, the difference was statistically significantbetween control smokers with normal lung functions (median ± IQR, 115±34 cells/mm^2)and severe/very severe COPD (median ± IQR; 193±37 cells/mm^2; Mann Whitney: p=0.030) (Fig 1. **Panel e**).

The expression of TLR4 was increased in the bronchial epithelium in severe/very severe COPD compared to control non-smokers (epithelial score 0-3: median ± IQR; 1 ± 0.1 vs. 1.75 ± 0.6; Mann Whitney: p=0.027) and tended to be

significant when compared to control smokers with normal lung function 1.25 ± 0.4 vs. 1.75 ± 0.6; Mann Whitney: p=0.054) (Table10; Fig. 2 panel f). In the lamina propria no differences were observed for TLR4 immunoexpression between severe/very severe COPD when compared to control non-smokers. However, the difference of TLR4+ cells was statistically significant between control smokers with normal lung functionand severe/very severe COPD (median ± IQR; 58 ± 21 vs. 111±51 cells/mm^2; Mann Whitney: p=0.027) and between control smokers and mild/moderate COPD (median ± IQR; 58 ± 21 vs. 99.5±42.5 cells/mm^2; Mann Whitney: p=0.015)(Fig 2. Panel e).

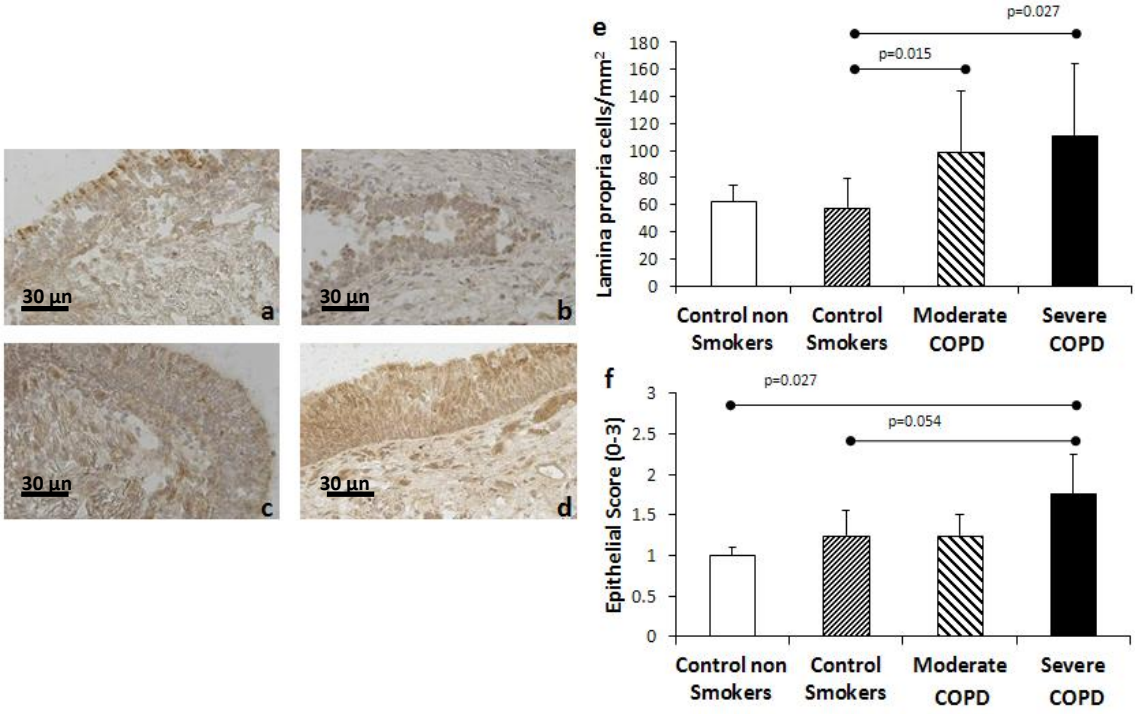


Fig 2: Photomicrographs showing the bronchial epithelium of a control non-smoker (panel a), control smokers (panel b), moderate COPD (panel c) and severe COPD patients(panel d) immunostained for identification of TLR4+ cells. Results are representative of those from 9 nonsmokers, 16 healthy smokers and 33 subjects with stable COPD. Internal scale bars = 30 µm. TLR4 positive cells in the lamina propria (panel e) and epithelium (panel f) of control non-smokers, control smokers and subjects with chronic obstructive pulmonary disease (Moderate COPD; Severe COPD). The results are expressed as the median ± IQR positive cells per millimetre of epithelium. Statistical analysis: Mann-Whitney U-test for comparison between groups. Nonsmokers: n=9; healthy smokers: n=16; COPD: n=33. *: p<0.05.

In the bronchial epithelium adapter Myd88 was increased with increasing severity of the disease. Myd88 expression was increased in severe/very severe COPD compared to control smokers (epithelial score 0-3: median ± IQR; 1.62 ± 0.22 vs. 2 ±

0.13; Mann Whitney: p=0.038) and mild/moderate COPD (median ± IQR; 1.5 ± 0.32 vs. 2 ± 0.13; Mann Whitney: p=0.012)(table10; Fig 3; panel f). No significant differences were observed for Myd88 immunoexpression in the four groups of subjects examined in the lamina propria (Fig 3; panel e).

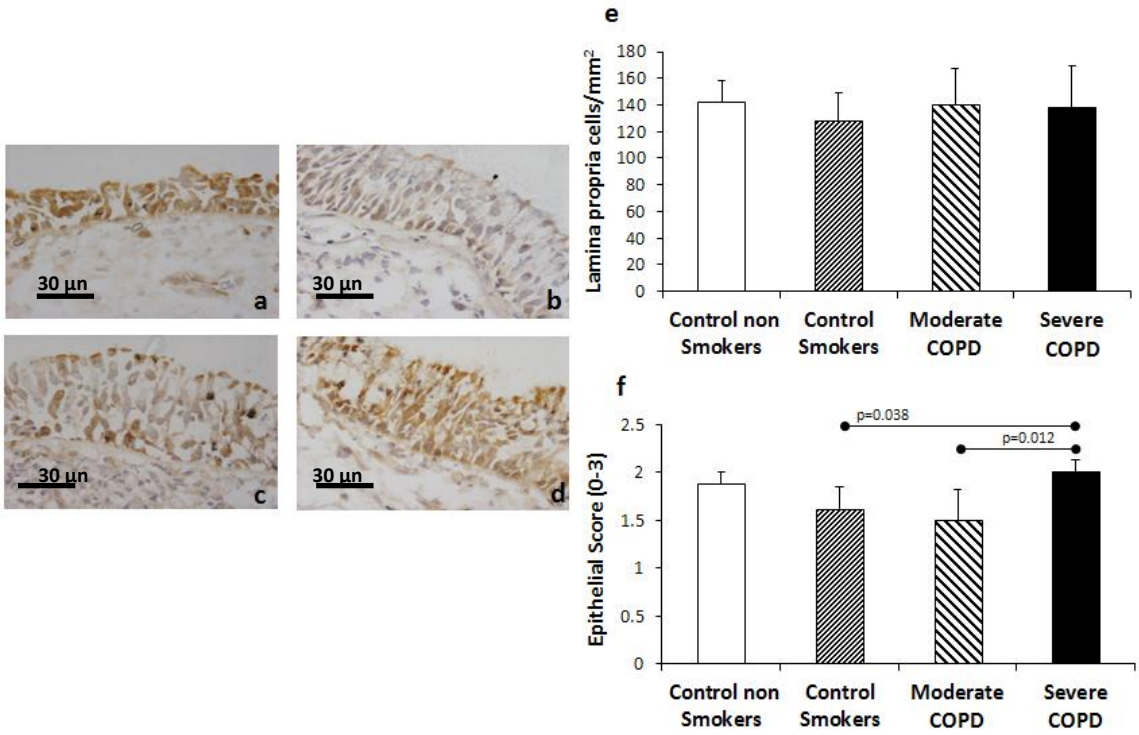


Fig 3: Photomicrographs showing the bronchial epithelium of a control non-smoker (panel a), control smokers (panel b), moderate COPD (panel c) and severe COPD patients(panel d) immunostained for identification of Myd88+ cells. Results are representative of those from 9 nonsmokers, 16 healthy smokers and 33 subjects with stable COPD. Internal scale bars = 30 µm. Myd88 positive cells in the lamina propria (panel e) and epithelium (panel f) of control non-smokers, control smokers and subjects with chronic obstructive pulmonary disease (Moderate COPD; Severe COPD). The results are expressed as the median ± IQR positive cells per millimetre of epithelium. Statistical analysis: Mann-Whitney U-test for comparison between groups. Nonsmokers: n=9; healthy smokers: n=16; COPD: n=33. *: p<0.05.

Some differences were observed in the bronchial epithelium between the four groups of subjects studied for all the other proteins investigated (table10).

Table 10: Bronchial epithelial scopre (0-3) used in immunohistochemistry

Protein	***Control non Smokers***	***Control Smokers***	***Mild/Moderate COPD***	***Severe COPD***	***p value* (Kruskal–Wallis)**
HSP60	0.7 (0.5-1.5)	1.2 (0.5-2.5)	1.7 (0.5-3.0	1.5 (0.5-2.5)#	**0.053**
TLR4	1.0 (0.75-2)	1.25 (0.5-2)	1.25 (0.75-2.25)	1.75(0.75-3)*&	**0.059**
Myd88	1.9 (1.25-2.5)	1.62 (0.75-2.5)	1.5 (0.75-2)	2 (0.75-2.5)§&	**0.050**
p-p38	0.75(0.25-1)	0.75(0.25-1.25)	0.5(0.25-1.25)	0.5 (0.25-1.5)	0.618
p-JNK1	0.12 (0-0.25)	0.1 (0-1)	0.12 (0-1)	0.25 (0-1)	0.521
pERK1/2	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	NA
IL-8	1.5 (0.5–2.5)	1.5 (1.0–1.5)	2.0 (1.5–2.5)	2.5 (1.5–2.5)&	0.32
IL-10	0.03 (0-0.25)	0.02 (0-0.25)	0.07 (0-0.5)	0.11 (0-0.5)	0.515

Abbreviations: COPD, chronic obstructive pulmonary disease, NA= not applicable. Data expressed as median (range). Statistics: the Kruskal-Wallis test was used for multiple comparisons followed by Mann-Whitney U test for comparison between groups: *p<0.05, significantly different from control non-smokers;&p<0.05, significantly different from control smokers with normal lung function; The exact "p" values for comparison between groups are given in the Results section.

4.2 IN-VITRO

4.2.1 IL-8 and IL-10 mRNA expression in bronchial epithelial cells (16HBE) after HSP60, H_2O_2, Cytomix, and LPS stimulation

The first experiments were designed to assess the activity of HSP60 and to examine the influence of oxidative, pro-inflammatory and bacterial stress on IL-8 and IL-10 mRNA production from bronchial epithelial cells (16-HBE). To this purpose 16-HBE were stimulated with HSP60 (1 μg/mL), H_2O_2 (100 μM), Cytomix (IL-1β 1 ng/mL, TNFα 10 ng/mL and INF-γ 10 ng/mL) and LPS (10 μg/mL) respectively, for 1, 2and 4h. A preliminary study has planned the exposure of 16HBE even at 8 and 24 hours; these data will not be shown as not significant. IL-8 and IL-10 mRNA expression was examined by RT-PCR. As shown in Fig. 1 (**panel a**), Hsp60 showed an anti-inflammatory effects, its stimulus down-regulated IL-8 mRNA expression after 2 and 4h of exposure (Non-Treated vs. Treated, mean ± SD: 0.81 ± 0.31 vs 0.19 ± $0.0072^{-\Delta\Delta Ct}$; p=0.009; 0.93 ± 0.19 vs 0.30 ± $0.122^{-\Delta\Delta Ct}$; p=0.001, respectively) and up-regulated IL-10 mRNA expression after 2h of exposure (Non-Treated vs. Treated, mean ± SD: 0.40 ± 0.04 vs 13.62 ± $5.39^{2-\Delta\Delta Ct}$; p=0.002) (Fig. 2;**panel a**).

The oxidative stress (H_2O_2) up-regulated IL-8 mRNA expression after 2h of exposure (Non-Treated vs. Treated, mean ± SD: 1.18 ± 0.30 vs 3.32 ± $0.472^{-\Delta\Delta Ct}$; p<0.0001) (Fig. 1; **panel b**). The Cytomix confirmed a strong pro-inflammatory activity, showing up-regulation of IL-8 after 1, 2 and 4h of exposure (Non-Treated vs. Treated, 1h mean ± SD: 2.84 ±0.29 vs 39.03 ± $12.812^{-\Delta\Delta Ct}$; p<0.0001; 2h mean ± SD: 1.18 ± 0.30 vs 7.63 ± $3.972^{-\Delta\Delta Ct}$; p=0.008; 4h mean ± SD: 0.32 ± 0.09 vs 2.24 ± $1.092^{-\Delta\Delta Ct}$; p=0.003) (Fig. 1; **panel c).**The anti-inflammatory response (IL-10mRNA) was not significantly changed between untreated cells versus treated cells after both H_2O_2 and Cytomix stimuli.LPS caused up-regulation of IL-8 mRNA after 2h and 4h (Non-Treated vs. Treated, mean ± SD: 1.13 ± 0.36 vs 1.96 ± 0.44 $2^{-\Delta\Delta Ct}$; p=0.023; 0.75 ± 0.16 vs 1.64 ± $0.572^{-\Delta\Delta Ct}$; p=0.025, respectively) (Fig.1; **panel d**). It is interesting to note that after 1h of treatment with LPS the IL-10 mRNA was significantly up-regulated (Non-Treated vs. Treated, mean ± SD: 1.00 ± 0.52 vs 3.27 ± 1.10 $2^{-\Delta\Delta Ct}$; p=0.008)(Fig. 2;**panel b**).

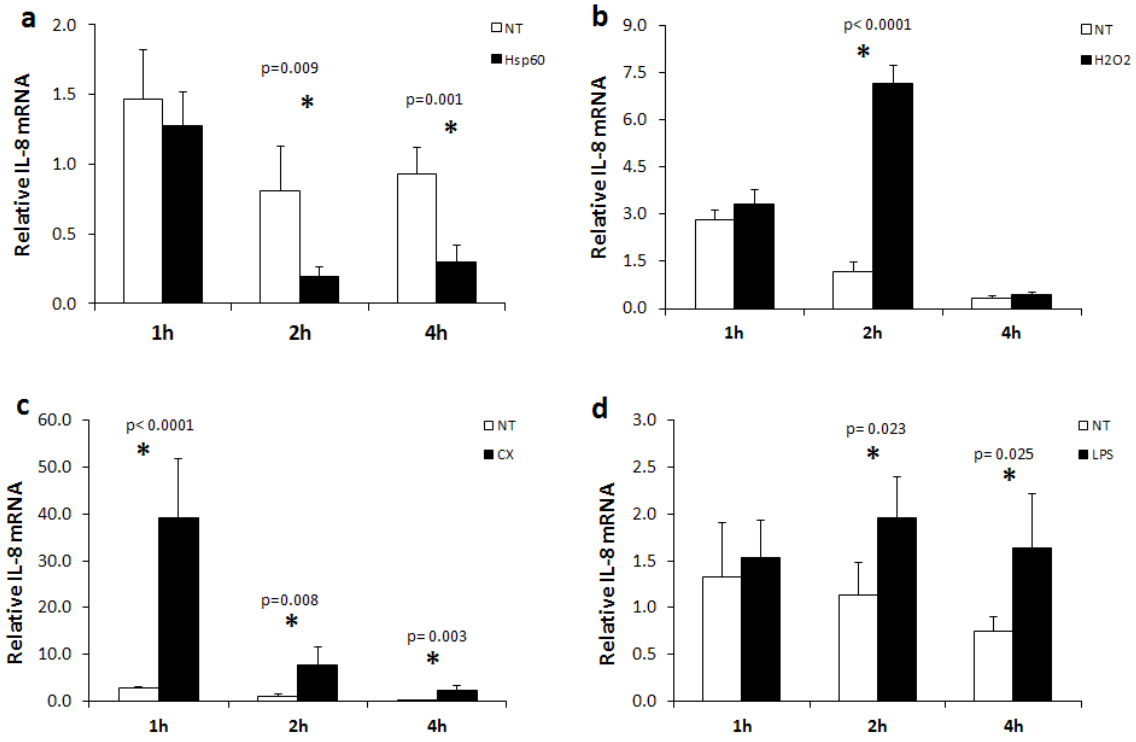


Fig 1: In vitro expression of IL-8 (CXCL8) mRNA in 16HBE cells treated with Hsp60 (1µg/mL) (Panel a),H2O2 (100µM) (Panel b), Cytomix (TNFα, 10 ng/ml, IL-1β, 1 ng/ml and IFNγ, 10 ng/ml) (Panel c) and Lipopolysaccharide (LPS) (Panel d) from P. aeruginosa.Treatments with Hsp60show IL-8 mRNA expression down-regulated at 2h (p=0.009) and 4h (p=0.001). All the other treatments up-regulated IL-8 mRNA expression at 2h. The Cytomix confirmed a strong pro-inflammatory activity, showing up-regulation of IL-8 after 1, 2 and 4 hours of exposure. All experiments were performed in quadruplicate. The expression levels of all genes studied were normalized to GAPDH levels in each sample to determine the expression between treated and non-treated cells using the $2^{-\Delta\Delta Ct}$ method (Livak KJ, 2001).Statistical analysis: T-test (*p<0.05) for comparison between treated and non-treated cells.

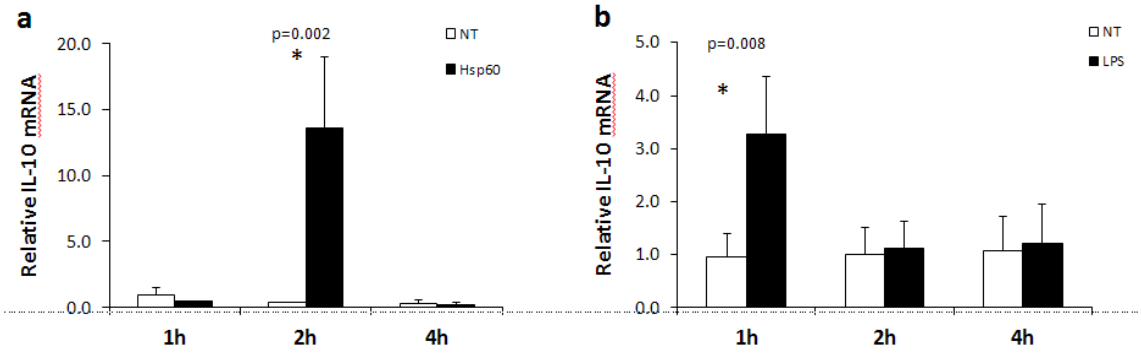


Fig 2: In vitro expression of IL-10 mRNA in 16HBE cells treated with Hsp60 (1µg/mL) and Lipopolysaccharide (LPS) from P.aeruginosa. Treatments with Hsp60 up-regulated IL-10 mRNA at 2h (p=0.002) (panel a). This may suggests an anti-inflammatory effect of HSP60. The anti-inflammatory response was not significantly changed after H_2O_2 and Cytomix (Graphs not shown). Interesting to note after 1h of treatment with LPS (Panel b), IL-10 was significantly up-regulated (p=0.008). All experiments were performed in quadruplicate. The expression levels of all genes studied were normalized to GAPDH levels in each sample to determine the expression between treated and non-treated cells using the $2^{-\Delta\Delta Ct}$ method (Livak KJ, 2001). Statistical analysis: T-test (* p<0.05) for comparison between treated and non-treated cells.

In addition, we investigated the main MAP-Kinase directly involved in the cytokines and chemokines transcriptional pathways such as NF-kb p65 subunit (p65) p38-α (MAPK14), JNK-1 (MAPK8) and ERK-1 (MAPK1) after inflammatory stress (Cytomix), oxidative stress (H_2O_2), bacterial stress (LPS), and HSP60 stimulus on 16-HBE.The RT-qPCR analysis of JNK-1 and ERK-1 did not show any change in mRNA expression between Non-Treated and Treated cells with all the stimuli used, except after pro-inflammatory stimulation (Cytomix) where JNK1 was up-regulated after 1h of stimulation, being the only MAP-kinase involved in this study after Cytomixstimulus on bronchial epithelium (Non-Treated vs. Treated, mean ± SD: 1.20 ± 0.75 vs 3.80 ± 0.37 $2^{-\Delta\Delta Ct}$; p=0.0029)(Fig.3; **panel c**).

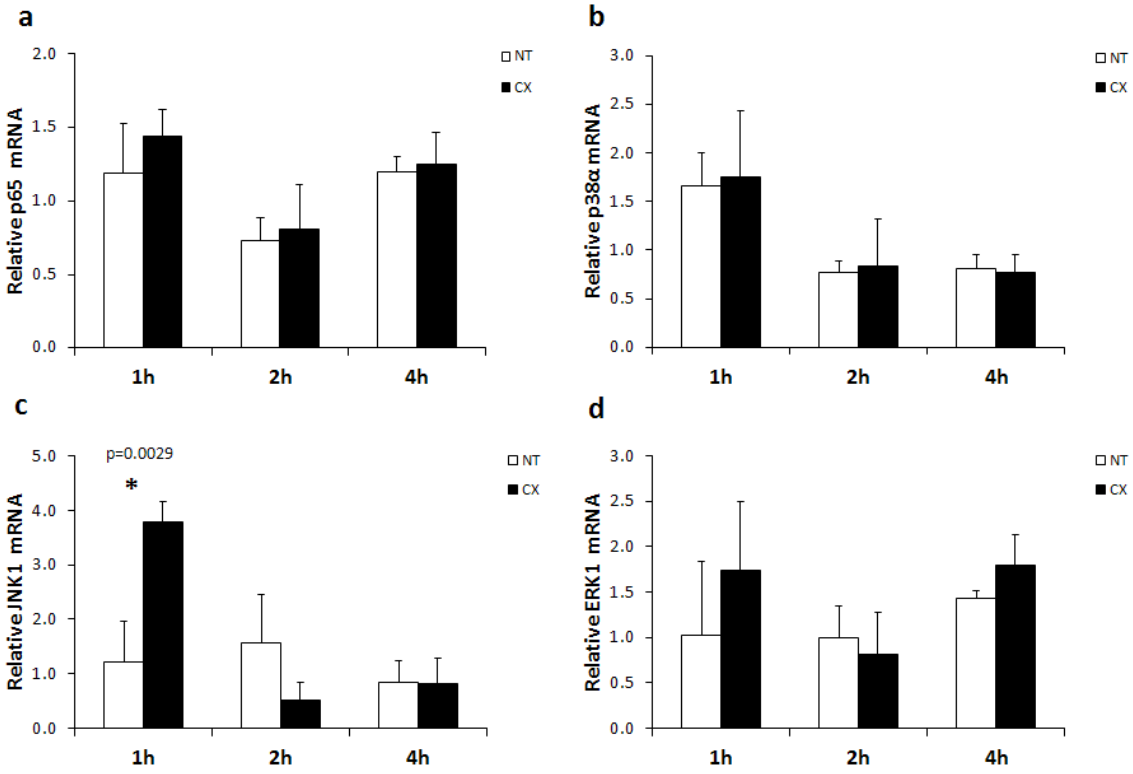


Fig 3: In vitro expression of NF-kB p65 subunit (panel a), p38α (panel b), JNK1(panel c) and ERK1 (panel d) mRNA in 16HBE cells treated with cytomix (TNFα, 10 ng/ml, IL-1β, 1 ng/ml and IFNγ, 10 ng/ml). Stimulation with pro-inflammatory cytomix is able to up-regulate only the MAPK JNK1 after 1h of stimulation Data expressed as mean ± Standard Deviation. All experiments were performed in quadruplicate.The expression levels of all genes studied were normalized to GAPDH levels in each sample to determine the expression between treated and non-treated cells using the $2^{-\Delta\Delta Ct}$ method (Livak KJ, 2001). Statistical analysis: T-test (*p<0.05) for comparison between treated and non-treated cells. The exact "p" values for comparison between groups are given in the graph.

However, the analysisofp38-α mRNA showed an increased expression after 2h of exposure toH_2O_2 (Non-Treated vs. Treated, mean ± SD: 0.77 ± 0.12 vs 1.07 ± 0.21 $2^{-\Delta\Delta Ct}$; p=0.048) (Fig. 4; **panel b**). While after stimulation with LPS, p38-α up-regulated

already after 1h of exposure (Non-Treated vs. Treated, mean ± SD: 0.70 ± 0.19 vs 1.60 ± 0.46 $2^{-\Delta\Delta Ct}$; p=0.002) (Fig. 5; **panel b**). This suggests an important role of the p38 MAPKα in the expression of cytokines and chemokines on bronchial epithelial cells. The Cytomix has not caused any variation of the p38-α mRNA level between Non-Treated and Treated samples(Fig. 3 **panel b**).

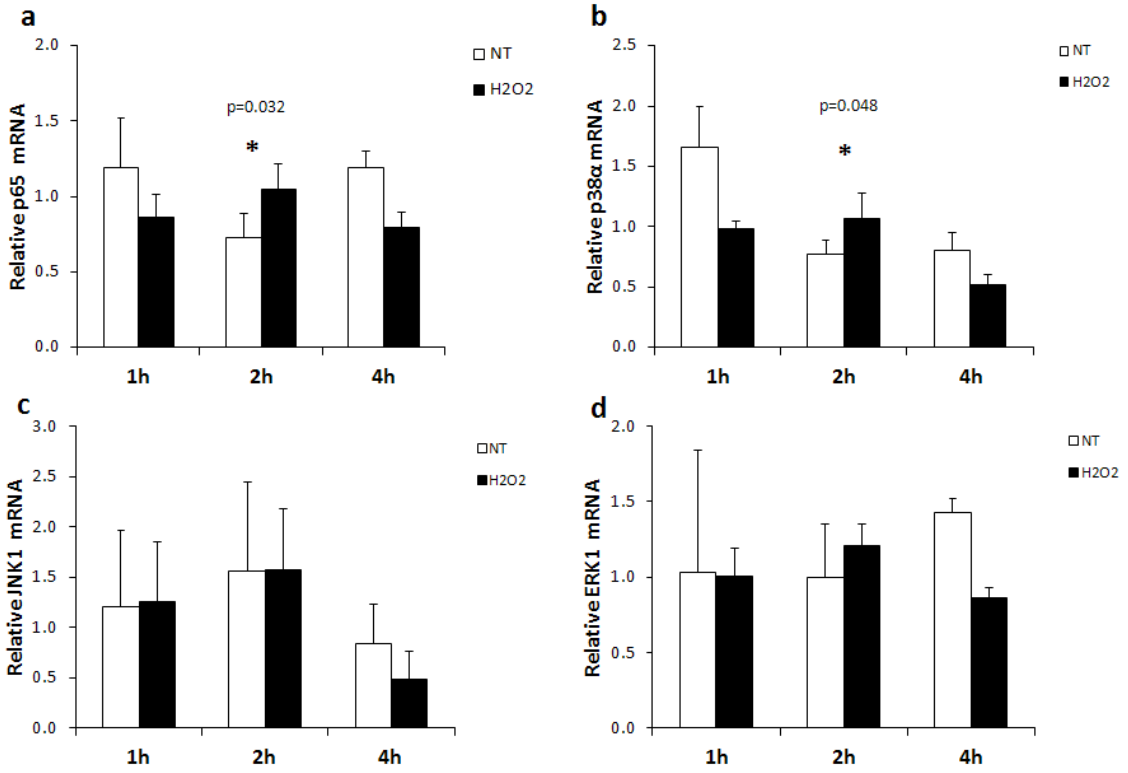


Fig 4: In vitro expression of NF-kB p65 subunit (panel a), p38α (panel b), JNK1(panel c) and ERK1 (panel d) mRNA in 16HBE cells treated with H_2O_2 (100µM). Oxidative stress up-regulated p38α (panel b) and p65 (panel e) mRNA at 2h after stimuli. All experiments were performed in quadruplicate. Data expressed as mean ±Standard Deviation. The expression levels of all genes studied were normalized to GAPDH levels in each sample to determine the expression between treated and non-treated cells using the $2^{-\Delta\Delta Ct}$ method (Livak KJ, 2001). Statistical analysis: T-test (*p<0.05) for comparison between treated and non-treated cells. The exact "p" values for comparison between groups are given in the graph.

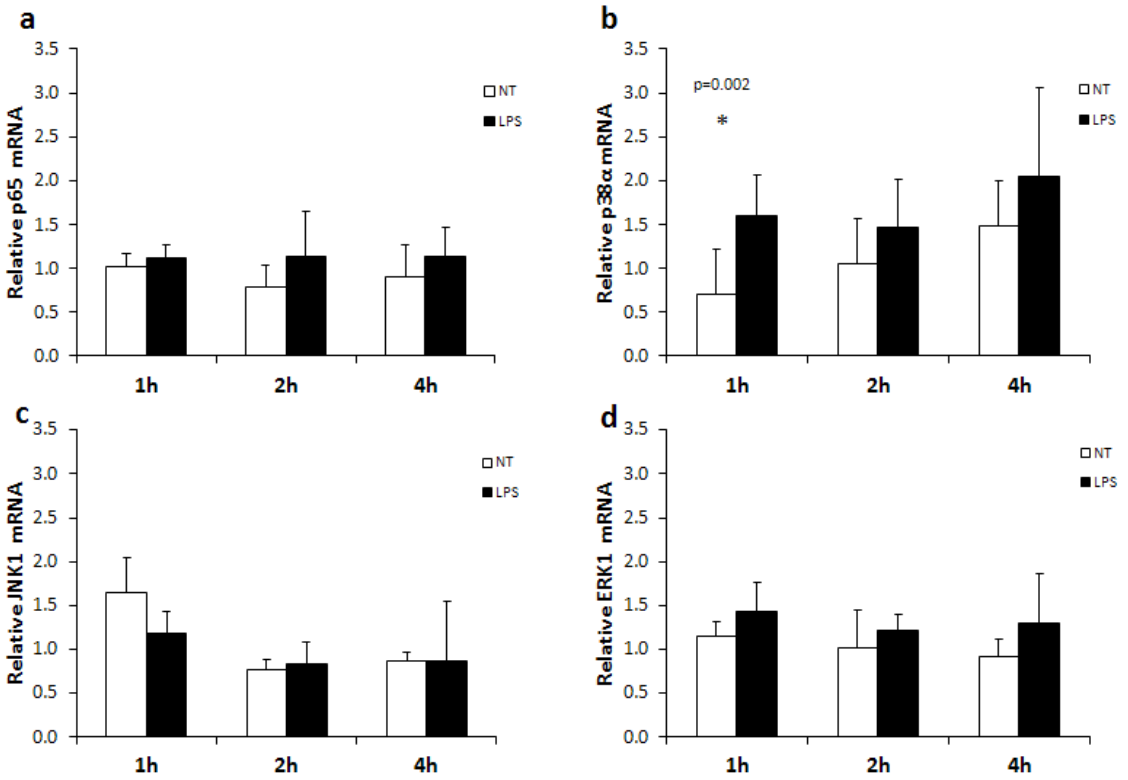


Fig 5: In vitro expression of NF-kB p65 subunit (panel a), pP38α (panel b), JNK1(panel c), ERK1 (panel d) mRNA in 16HBE cells treated with Lipopolysaccharide (LPS) from P. aeruginosa. Bacterial stimulus up-regulated p-38 (panel b) mRNA at 1h.MAPK p38 seems to be the only kinase involved in response to LPS on bronchial epithelial cells. All experiments were performed in quadruplicate. Data expressed as mean ± Standard Deviation. The expression levels of all genes studied were normalized to GAPDH levels in each sample to determine the expression between treated and non-treated cells using the $2^{-\Delta\Delta Ct}$ method (Livak KJ, 2001). Statistical analysis: T-test (*p<0.05) for comparison between treated and non-treated cells. The exact "p" values for comparison between groups are given in the graph.

NF-kB p65 subunit mRNA was significantly increased at 2h after H_2O_2(Non-Treated vs. Treated, mean ± SD: 0.73 ± 0.16 vs 1.05 ± 0.17 $2^{-\Delta\Delta Ct}$; p=0.032)(fig. 4; **panel a**),but it did not reach statistical significance after Cytomix (Fig. 3; **panel a**) and LPS (Fig. 5; **panel a**) stimulations.

4.2.2 HSP60 anti-inflammatory activity

To explore whether Human extracellular HSP60 is able to play an anti-inflammatory role on bronchial epithelial cells, 16-HBE cells were stimulated combining Hsp60 (1 µg/mL) with H2O2 (100 µM), with LPS (10 µg/ml) and with Citomix (IL-1β 1 ng/mL, TNFα 10 ng/mL and INF-γ 10 ng/mL) (Fig. 6).

The addition of HSP60 (1 µg/mL) to H_2O_2 (100 µM) significantly inhibited IL-8 mRNA expression at 2h of exposure (Non-Treated vs. Treated, mean ± SD: 1.28 ± 0.16

vs 1.08 ± 0.40 $2^{-\Delta\Delta Ct}$; p=0.8498). (Fig. 6; **panel a**). The addition of HSP60 (1 µg/mL) to LPS (10 µg/ml) inhibited IL-8 mRNA expression only after 4h of exposure (Non-Treated vs. Treated, mean ± SD: 0.99 ± 0.71 vs 0.70 ± 0.292$^{-\Delta\Delta Ct}$; p=0.4816) (Fig.6; **panel c**), confirming a cytoprotective effect on bronchial epithelium; after 2h of exposure HSP60 did not inhibited IL-8 mRNA expression (Non-Treated vs. Treatedmean ± SD: 0.67 ± 0.25 vs 1.75 ± 0.71 $2^{-\Delta\Delta Ct}$; p=0.0289). Addition of HSP60 (1 µg/mL) to Cytomix (IL-1β 1 ng/mL, TNFα 10 ng/mL and INF-γ 10 ng/mL) did not inhibit IL-8 mRNA expression at all exposure times and confirmed a strong pro-inflammatory activity (Fig.6 **panel b**).

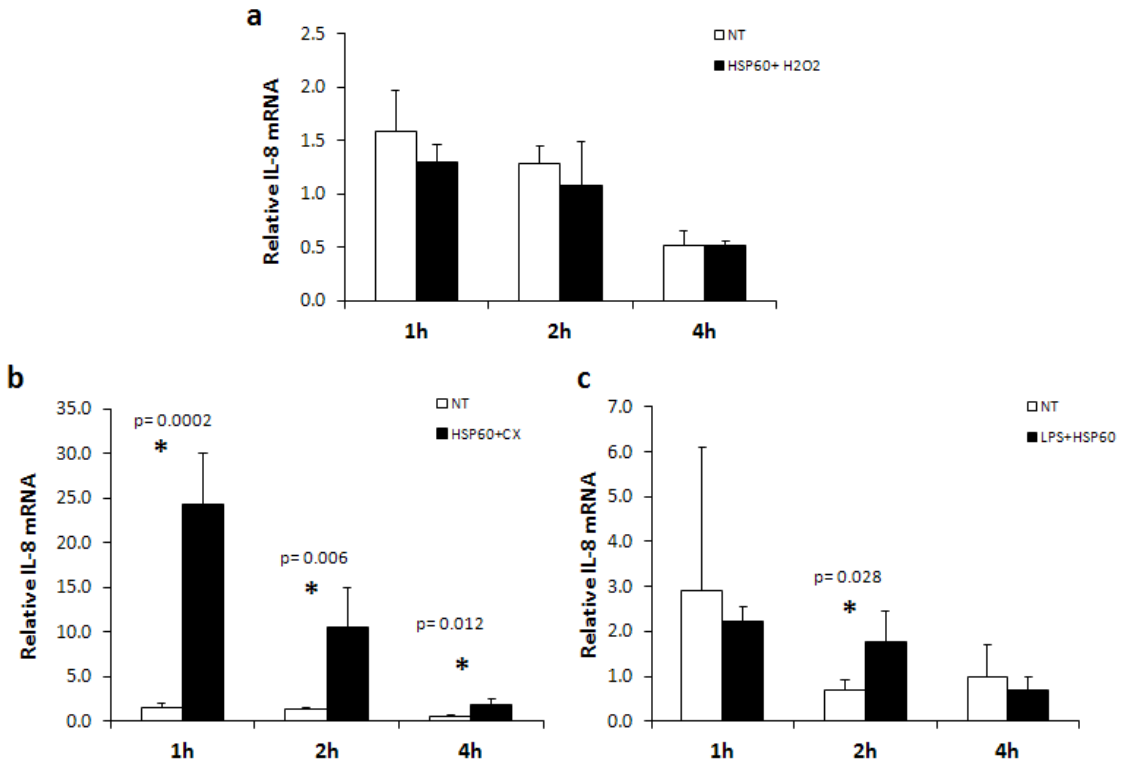


Fig. 6: In vitro expression of IL-8 in 16HBE human bronchial epithelial cells treated with combined Hsp60 (1 µg/ml) + H_2O_2 (100µM) (panel a), HSP60 (1 µg/ml) + Citomix (TNFα 10ng/ml, IL-1β 1 ng/ml and INFµ 10 ng/ml) (panel b) and Hsp60 (1 µg/ml) + LPS from P. aeruginosa (10µg/ml) (c). Addition of HSP60 (1 µg/mL) to H2O2 (100 µM) did not show any change in mRNA expression between Non-Treated and Treated samples (n=4, T test, p>0.05) (panel a) with a significant inhibition of IL-8 mRNA expression. Addition of HSP60 (1 µg/mL) to Cytomix (IL-1β 1 ng/mL, TNFα 10 ng/mL and INF-γ 10 ng/mL) did not inhibit IL-8 mRNA expression (n=4, T test, p>0.05) (panel b). The addition of HSP60 (1 µg/mL) to LPS (10 µg/ml) inhibited IL-8 mRNA expression only after 4 hours of exposure (n=4, T test, p>0.05), after 2 hours of exposure the mRNA expression of IL-8 is still significantly up-regulated (n=4, T test, p<0.05) (panel c).The expression levels of all genes studied were normalized to GAPDH levels in each sample to determine the expression between treated and non-treated cells using the $2^{-\Delta\Delta Ct}$ method (Livak KJ, 2001). Statistical analysis: T-test (* p<0.05) for comparison between treated and non-treated cells.

4.2.3 TLR4 expression pathway in bronchial epithelial cells (16HBE) after HSP60 stimulation

Extracellular HSP60 binds to numerous surface receptors, including TLR4. In this study we tried to understand the mechanisms and the possible signal pathways involved after HSP60 stimulation in lung epithelial cells (16-HBE). We quantified by qRT-PCR the mRNA levels of the main molecules and MAPKinase directly involved in the cytokines and chemokines transcriptional pathways such as Myd88, p38-α (MAPK14), ERK-1 (MAPK1),JNK-1 (MAPK8), NF-Kb p65 subunit and CREB1. Myd88 mRNA expression was significantly increased at 2h after exposure to HSP60 (Non-Treated vs. Treated, mean ± SD: 1.28 ± 0.42 vs 2.37 ± 0.47 ΔCt; p=0.014) (Fig. 7; **panel a**). Although p38α mRNA was significantly increased at 1h (Non-Treated vs. Treated, mean ± SD: 1.08 ± 0.31 vs 1.52 ± 0.16 $2^{-\Delta\Delta Ct}$; p=0.002) (Fig. 7; **panel b**), while JNK1 and ERK1 mRNA was not significantly changed after the treatment used. NF-kB p65 subunit mRNA was significantly increased after 2h ((Non-Treated vs. Treated, mean ± SD: 0.87 ± 0.19 vs 1.61 ± 0.44 $2^{-\Delta\Delta Ct}$; p=0.002) and 4h (1.81 ± $0.112^{-\Delta\Delta Ct}$; p=0.003,) of exposure (Fig. 7; **panel c**). This, once again, demonstrates the direct involvement of p65 subunit in the modulation of the inflammatory process. CREB1, the nuclear transcription factor involved in p38α Kinase pathway, was up-regulated at 1h and 4h after HSP60 stimulation (Non-Treated vs. Treated, mean ± SD: 1.14 ± 0.32 vs 3.19 ± $2.052^{-\Delta\Delta Ct}$; p=0.002; 1.09 ± 0.34 vs 7.21 ± $4.602^{-\Delta\Delta Ct}$; p=0.002, respectively) (Fig. 7; **panel d**).

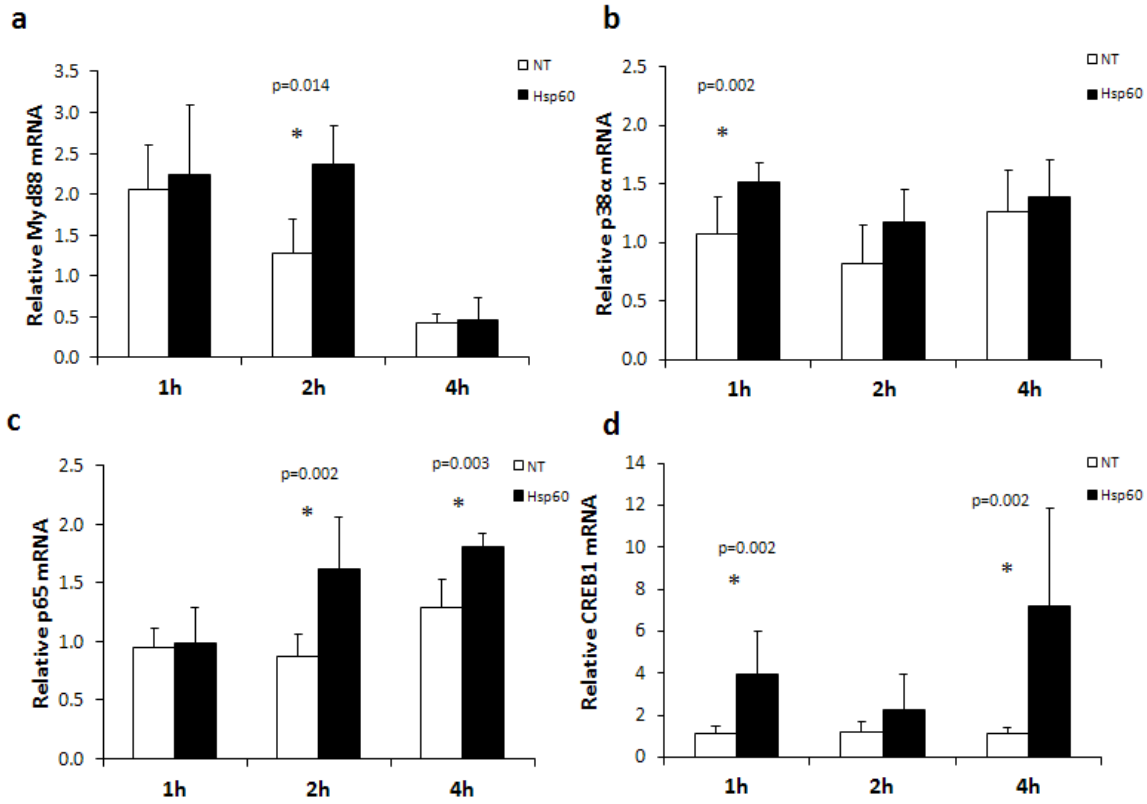


Fig. 7: In vitro expression of NF-kB p65 subunit (panel a), pP38α (panel b), ERK1(panel c) and CREB1(panel d) mRNA in 16HBE cells treated with human low endotoxic HSP60 (1 µg/ml). HSP60 stimulation up-regulated p65(panel a) mRNA at 2h and 4h after stimuli. P38α (panel b)mRNA was up-regulated already after 1h of stimulations and CREB1 was up-regulated at 1h and 4h after the exposure. All experiments were performed in quadruplicate. Data expressed as mean ± Standard Deviation. The expression levels of all genes studied were normalized to GAPDH levels in each sample to determine the expression between treated and non-treated cells using the $2^{-\Delta\Delta Ct}$ method (Livak KJ, 2001). Statistical analysis: T-test (*p<0.05) for comparison between treated and non-treated cells. The exact "p" values for comparison between groups are given in the graph.

4.2.4 Effect of p38alpha kinase and TLR4 inhibition on 16HBE after H_2O_2, LPS and HSP60stimulation forIL-8 and IL-10 production.

Since p38α, among the MAP kinases here studied, was the one most expressed in both "ex vivo" bronchial biopsies analysis and also after oxidative and LPS stimuli in "in vitro" experiments, we investigated the role of p38α activation in relation to IL-8 over-production after H_2O_2 or LPS stimulation. To this end, we performed a 30-min pre-treatment of cultured 16HBE cells with p38 MAPK inhibitor (SB203580; 10 µM)followed by 1h, 2h and 4h H_2O_2 or LPS stimulation. Blocking p38α activity, IL-8 mRNA production was not changed at 1h, 2h and 4h after H_2O_2 (Fig. 8; **panel a**) or LPS (Fig. 8; **panel b**) challenge.

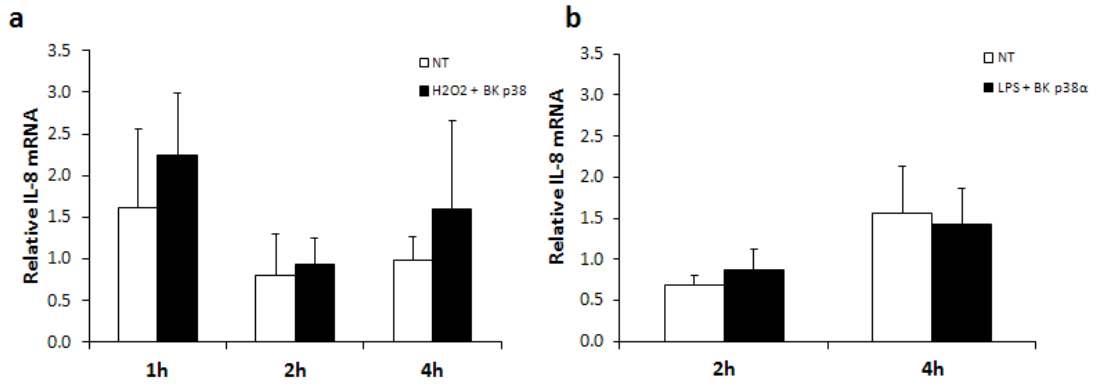


Fig. 8: In vitro expression of IL-8 (CXCL8) (panel b) mRNA after a 30-min pre-treatment with a p38 inhibitor (SB203580; 10 µM) followed by treatmentwith H_2O_2(panel a)and lipopolysaccharide (LPS) from P.aeruginosa (panel b). P38 inhibitor reduced the IL-8 mRNA up-regulation observed at 2h and 4h after LPS stimulation and inhibitedmRNA expression after 2h of exposure with H_2O_2. All experiments were performed in quadruplicate.The expression levels of all genes studied were normalized to GAPDH levels in each sample to determine the expression between treated and non-treated cells using the $2^{-\Delta\Delta Ct}$ method (Livak KJ, 2001). Statistical analysis: T-test (* p<0.05) for comparison between treated and non-treated cells.

P38 inhibitor reduced the IL-10and IL-8 mRNA up-regulation previously observed (fig. 9 **panel b**) and inhibited the CREB1 mRNA expression after 1h and 4h of exposure (fig.9 **panel a**).

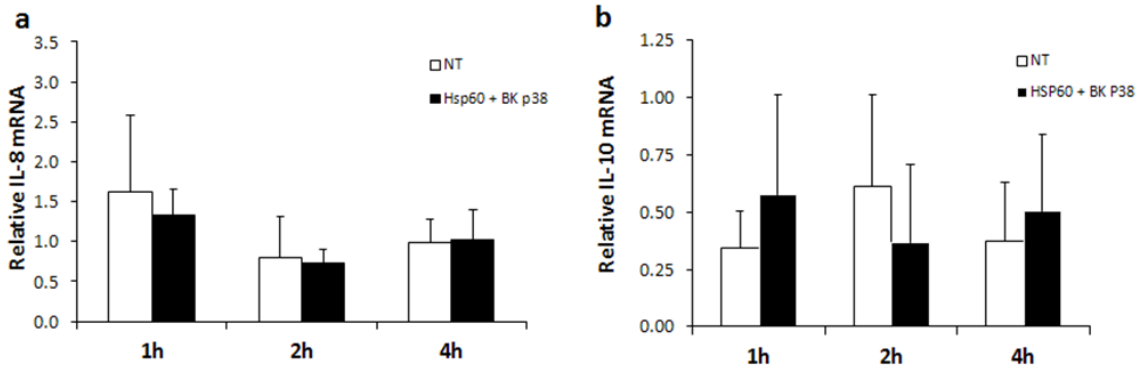


Fig. 9: In vitro expression of (CXCL8) (panel a) and IL-10 (panel b) mRNA after a 30-min pre-treatment with a p38 inhibitor (SB203580; 10 µM) followed by treatmentwith human low endotoxic HSP60 (1µg/mL). P38 inhibitor reduced the IL-10 mRNA up-regulation observed at 2h after HSP60 stimulation and inhibits IL-8mRNA down-regulation after 2hand 4h of exposure. All experiments were performed in quadruplicate. The expression levels of all genes studied were normalized to GAPDH levels in each sample to determine the expression between treated and non-treated cells using the $2^{-\Delta\Delta Ct}$ method (Livak KJ, 2001.) Statistical analysis: T-test (* p<0.05) for comparison between treated and non-treated cells.

In addition, we studied the effect of TLR4 inhibition on 16HBE after LPS stimulation to assess IL-8 mRNA production. The TLR4 was observed to be the main cellular receptor of Gram negative bacteria. When activated it is involved in the activation of multiple pathways for the transcription of important cytokines and chemokines such as IL-8, TNF-alpha and IL-1beta. In this research, we wanted to inactivate the action of TLR4 by a molecular competitor blocker (LPS-RS Ultrapure, InvivoGen Italy) with the

purpose to evaluate the expression of mRNA for IL-8. 16HBE cells were treated with the TLR4 inhibitor (LPS-RS Ultrapure) 100 µg/mL together with LPS (10 µg/mL). Real time PCR analyses demonstrated a significant inhibition of IL-8 mRNA production both at 2 and at 4 hours of exposure (Fig. 10) (Non-Treated vs. Treated, mean ± SD: 2.36 ± 1.57 vs 2.09 ± 0.32 $2^{-\Delta\Delta Ct}$; p=0.4236; 2.49 ± 0.07 vs 2.32 ± 0.112$^{-\Delta\Delta Ct}$; p=0.8564, respectively). This finding confirmed that TLR4 is an important receptor on bronchial epithelial cells, and that its activation is involved in IL-8 mRNA production and release.

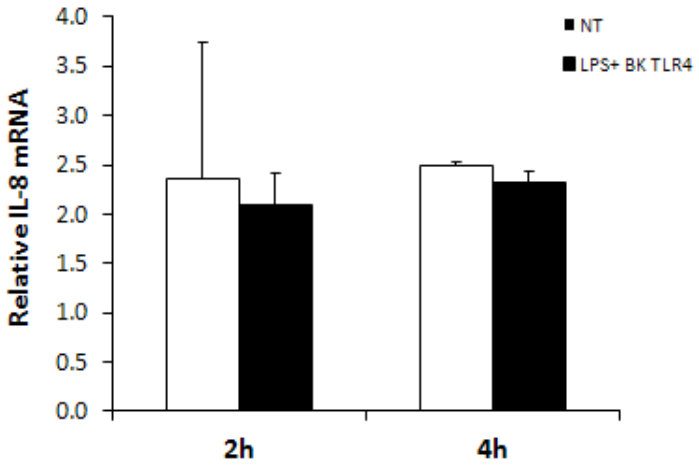
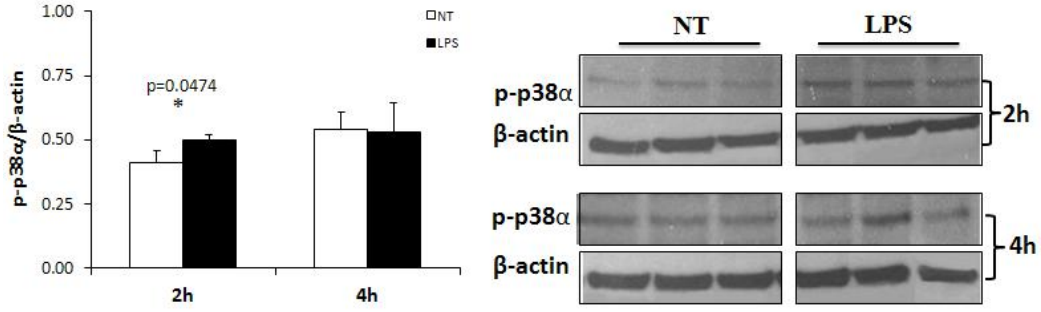


Fig. 10: In vitro expression of IL-8 (CXCL8) mRNA after (LPS-RS Ultrapure) 100 µg/mL together with LPS (10 µg/mL) from P.aeruginosa stimulation. TLR4 inhibitor reduced the IL-8 mRNA up-regulation observed at 2h and 4h after LPS stimulation. All experiments were performed in quadruplicate. The expression levels of all genes studied were normalized to GAPDH levels in each sample to determine the expression between treated and non-treated cells using the $2^{-\Delta\Delta Ct}$ method (Livak KJ, 2001.) Statistical analysis: T-test (* p<0.05) for comparison between treated and non-treated cells.

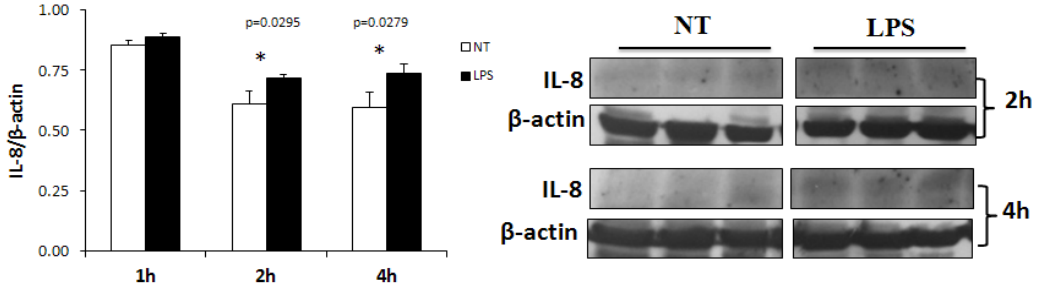
4.2.5 Western blotting for p-p38 MAPK and IL-8 in 16-HBE after LPS, H_2O_2 and HSP60 stimulation

Human bronchial epithelial cells were stimulated with LPS, H_2O_2 and HSP60 for "in vitro" Western blotting analysis to evaluate the intracellular p-p38α and IL-8 protein concentration. Significant differences were observed for p-p38 MAPK/actin ratio between non-treated (NT) and treated cells after 2h stimulation with LPS (mean± SD:0.408± 0.029vs 0.497± 0.012;p=0.0474)(Fig. 11, **panel a**). In fig. 11 **panel b** is reported theIL-8 protein up-regulation after 2h and 4h of LPS stimulation (Non-Treated vs. Treated, mean ± SD: 0.61 ± 0.05 vs 0.72 ± 0.02; p=0.0295; 0.60 ± 0.06 vs 0.74 ± 0.04; p=0.0279, respectively). These findings confirm the mRNA data shown above and the pro-inflammatory effect of LPS on 16-HBE.Oxidative stress confirms also what already observed with mRNA analysis: an up-regulation of the intracellular protein IL-8 at 2h (mean ± SD: 0.61 ± 0.05 vs 0.73 ± 0.004; p=0.0190). IL-8 protein after stimulation of epithelial cells with HSP60 confirmed that there is no variation between treated and non-treated cells (Fig. 11. **Panel c**). This once again confirms what already observed with the analysis of mRNA.

a



b



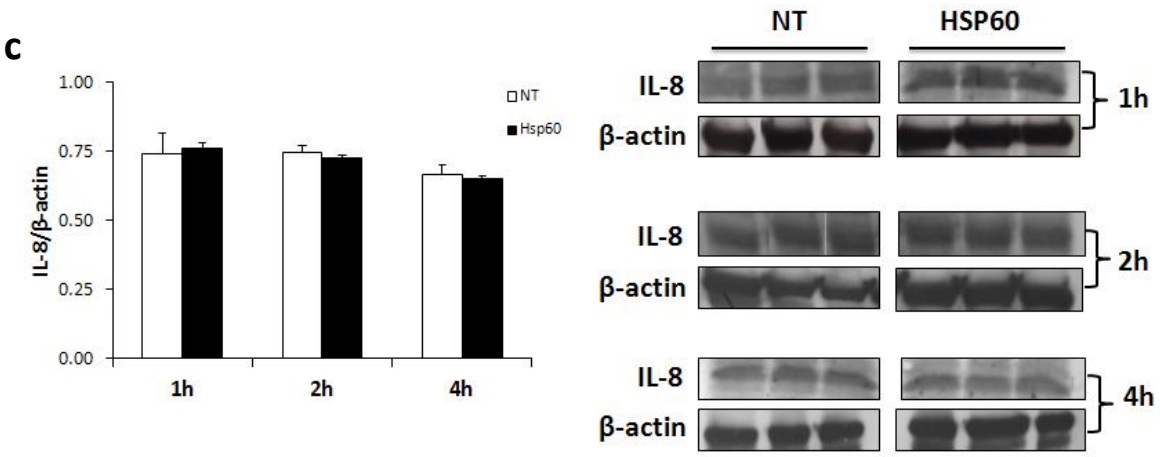


Fig. 11: Western blot analysis of activated phospho-p38MAPK (panel a) and IL-8 (panel b) after LPS stimulation "in vitro" bronchial epithelial cells normalized with β-actin as control for loading. A graphical analysis of the densitometric ratio of p-p38MAPK/β-actin is shown in panel a. It shows an up-regulation of intracellular protein after 2h with LPS stimulation; even the IL-8 intracellular protein confirms the data obtained previously with the mRNA, confirming its up-regulation at 2h and 4h of LPS exposure (panel b). In panel c is reported the data of IL-8 protein after stimulation with HSP60 (1 µg/ml); the densitometric ratio of IL-8/β-actin did not show any variation in the production of IL-8, confirming the possible non-inflammatory effect of HSP60.

5 Discussion

COPD is a lung disease characterized by chronic airflow limitation not fully reversible. This airflow limitation is usually progressive and is associated with an abnormal inflammatory response of the lung in both central (bronchi) and peripheral (bronchioles) airways (Barnes; 2000, Di Stefano et al; 2004, Hogg et al; 2004, GOLD; 2013). Today, COPD is considered one of the most important chronic inflammatory diseases due to its impact not only on human health but also on global economy. Unfortunately an effective treatment of the inflammatory state on airway damage and remodeling processes has not been still found. Several types of stressors overwhelm lungs during all the life of COPD patients. These stressors may determine damages to cell components, among which structural proteins and enzymes. Lungs are continuously exposed to oxidizing substances such as environmental pollutants or cigarette smoke and pathogenic microorganisms. In recent years, many studies are concentrated on the possible role of epithelial cells. The bronchial epithelium performs an important role in the response to pathogens and oxidative stress, with production of pro-inflammatory cytokines (Ling Ye et al., 2009). The surface of the airway epithelium represents a battleground in which the host intercepts signals from pathogens and activates epithelial defences against infections.

In the study presented in this thesis weanalyzed bronchial biopsies of 55 subjects: 32 with a diagnosis of COPD in a stable clinical stage, 12 smokers or ex-smokers with normal lung function and 11 non-smokers with normal lung function. The immunohistochemistry analysis has allowed to evaluate the expression at the level of bronchial epithelium and lamina propria of molecules involved in response to different inflammatory stimuli. In particular, it has been studied the TLR4 pathway. TLR4 is the main receptor of the innate immune system involved in the recognition of compounds derived respectively from gram positive (peptidoglycan and lipoteichoic acid) and gram negative bacteria (lipopolysaccharide). Moreover, we evaluated the role of TLR4 as a key receptor for extracellular HSP60 challenge (Chen et al; 1999). On the basis of these considerations, we observed that TLR4 was increased in biopsies of severe COPD patients compared to other groups of studied subjects. Especially in bronchial epithelium where TLR4 levels are significantly increased in patients with severe COPD compared with control smokers and controls non-smoker subjects. These date are in contrast with several studies suggesting that TLR4 expression in epithelial cells did not differ significantly between COPD patients and normal subjects (Nadigel J, 2011). In nasal epithelium MacRedmond et al. (2007) showed a reduction of TLR4 expression

with the increase of COPD severity. Lee et al. (2012) concluded their study by stating that TLR4 down-regulation in the lung was associated with emphysema and airflow limitation in smokers. Furthermore, most studies have suggested that TLR4 expression is decreased in the blood of COPD patients (Knobloch J, 2011) and TLR4 mRNA expression in induced sputum showed no significant difference between COPD patients and healthy controls (Baines KJ, 2011). Furthermore, it is known that the TLR4 signals may act through two different pathways, a Myd88-dependent and a Myd88-independent signaling pathway. Myd88 is a fundamental adapter of TLR4, and Myd88-dependent signaling cascade results in the production of pro-inflammatory cytokines through activation of NF-kB and MAPK (Akira S et al., 2006). IHC analysis showed increased expression of Myd88 in the epithelium of patients with severe COPD. This may suggests that in the bronchial epithelium of patients with COPD the Myd88-dependent pathway could be involved in the activation of the inflammation process. In the lamina propria were have not found differences in Myd88 expression between studied groups.

The increase of TLR4 in bronchial biopsies may be a direct consequence of the predominance of gram negative bacteria in the airways of these patients. Patients considered in this study had a stable COPD. It is known that the bacterial load increases further during exacerbations and that the restoration of a lower level of bacterial load by antibiotic treatment is related to a state of relative welfare with a reduction of airway inflammation in COPD patients who return to a stable clinic condition. Therefore, it would be particularly useful to establish "cut-off" values of bacterial concentrations that can trigger stages of disease exacerbation and better define the innate immune response that corresponds to these bacterial loads in the bronchi of these subjects. With this study we have begun to define the prevalence and type of anti-bacterial immune response that develops in patients with COPD. The up-regulation of Myd88, found in bronchial biopsies, enhances the role of the Myd88-dependent signaling pathway as the main way used by bronchial epithelium in response to bacterial stimuli.

In order to increase our understanding of the molecules involved in the TLR4-Myd88 pathway, we have also analyzed the main MAP-kinases involved. We observed a similar immune-expression of phospho-p38 MAPK, p-JNK1 and p-ERK1/2 in the bronchial epithelium and lamina propria of patients with mild/moderate and severe stable COPD when compared to control non-smokers. However, in patients with COPD phospho-p38MAPK immunopositivity was increased in the small airway epithelium when compared to control non-smokers (Gaffey K. et al., 2013) and in alveolar septa

when compared to control smokers and non-smokers (Renda T. et al., 2008). Phospho-p38 MAPK immunopositivity in lymphocytes populating the lamina propria of peripheral airways of COPD patients was similar to that found in control smokers and non-smokers (Gaffey K. et al., 2013). These last data are in part in agreement with our present observations of no changes of p-p38 MAPK immunopositivity in the bronchial biopsy lamina propria of stable COPD patients compared to control smokers and non-smokers.

Another study of our research group has shown that in the bronchial biopsies of smokers with normal lung function and in smokers with mild/moderate COPD there is an increase in the expression of NF-kb p65 subunit protein in comparison with bronchial biopsies from control non-smokers (Di Stefano et al., 2002). This was due to increased numbers of p65 positive epithelial cells in comparison to control non-smokers, as shown by immunohistochemistry analysis. Furthermore, in subjects with mild-to-moderate COPD, the number of p65 epithelial cells is increased by 100% and significantly correlated with the degree of airflow limitation. This suggested that changes in the epithelial p65 subunit expression may be associated with the state of the disease. For this reason it has been speculated that repeated bouts of viral and bacterial origin associated with TLRs response and oxidative stress, may induce a cascade of events resulting in NF-kB induction and activation, cytokine and chemokine production and further inflammatory cell infiltration. Based on these considerations, we investigated also the levels of pro-inflammatory IL-8 and anti-inflammatory IL-10 cytokine involved in COPD.The IHC analysis on bronchial biopsies showed that both IL-8 and IL-10 did not change significantly between the study groups examined in bronchial epithelium,but both showed an increasing trend with increasing disease severity. However, it is interesting to note that the IL-8protein was significantly increased only in bronchial epithelium of patients with severe/very severe COPD compared with control healthy smokers ($p=0.022$, Mann-Whitney U test). Further investigation with both Western blot analysis and RT-PCR did not show any variation between patients with stable COPD compared to controls smokers and non-smokers with normal lung function.These data are in line with other authors who have found increased IL-8 levels only in blood and sputum of patients with COPD exacerbations (Yamamoto C., 1997; Vaitkus M., 2013).Further studies would be important on exacerbated patients but unfortunately, collection of bronchial biopsies in COPD

subjects during exacerbations is difficult to perform, rarely accepted by the patients and not allowed by Ethical Committees

To better understand whether the exposure to several pro-inflammatory stimuli alter the normal response of the bronchial epithelium, in the present study we created an experimental model using human bronchial epithelium.We demonstrated above that TLR4 expression is up-regulated in severe COPD patients compared with control smokers and controls non-smokers. For this reason we wanted to investigate the ability of TLR4 to bind LPS and to direct the activation toward an increased release of IL-8. Furthermore, we treated the bronchial epithelium with H_2O_2 (100 μM) and cytomix (IL-1β 1 ng/mL, TNFα 10 ng/mL and INF-γ 10 ng/mL) to investigate the oxidative and pro-inflammatory stress, respectively. Our experiments show an up-regulation of IL-8 mRNA expression after all the stimuli used. This was associated with up-regulation of p38α MAPK mRNA after H_2O_2 and LPS but not cytomix exposure. These date are in agreement with other "in vitro" studies where using P.*aeruginosa,* as challenge factor, on human bronchial epithelial cells, it has shown an increased phosphorylation of p38 MAPK and IL-8 gene expression which was reduced by the use of p38 inhibitors (Bezzerri V. et al., 2011). Flagellin from P.*aeruginosa* increased the expression of IL-8 in BEAS-2B cells compared to untreated cells and addition of p38 MAPK inhibitors reduced IL-8 expression (Yang JJ., 2011). IL-8 mRNA and protein expression was also increased after LPS stimulation of bronchial epithelial cells (Zhang JX et al., 2012). Furthermore, stimulation with hydrogen peroxide increased IL-8 gene expression in BEAS-2B cells (Durham AL et al., 2013). In our model oxidative stressalso increaseNF-kB p65 mRNA: this confirms what was already reported by some authors in an "ex vivo" study, showing the activation of the transcription factor (NF-κB) in bronchial epithelium in response to oxidative stress (Di Stefano et al., 2002; Karin M, 2009). Cytomix stimulus, followed by IL-8 mRNA increase, was associated only to an increase of JNK1mRNA. This confirms what has already been demonstrated by Guo Y. et al. (1998): TNF-α induces a transient activation of JNK.Our data suggest that in bronchial epithelial cells (16-HBE) the p38 MAPK pathway may be more relevant after LPS and oxidative (H_2O_2) stimulation. Furthermore, pre-treatment of H_2O_2 and LPS exposed 16HBE cells with a p38 MAPK inhibitor, abolished the IL-8 mRNA increase, confirming the involvement of this MAPK kinase in IL-8 mRNA up-regulation. These date are in contrast with Pace E. et al. (2008) who claim that the main MAPKs involved in the up-regulation of IL-8 mRNA are ERK1/2 after stimulation with LPS on 16-HBE

and with Wang X. et al. (2014) that have recently shown on BEAS-2B the activation of ERK and JNK after LPSstimulation.

To confirm the involvement of the p38-MAP-kinase we performed a Western blot assay using a specific antibody directed against the phosphorylated form of p38. The protein analysis validated the data showing an increase of the activated protein at 2h after stimulation with LPS. Furthermore, intracellular IL-8 was increased in treated cells compared to untreated cells, confirming once again the key role of the p38-MAP-kinase. Western blot assay also confirmed that the intracellular IL-8 protein was increased even after oxidative stress challenge. These data are in agreement with the data obtained by Roos-Engstrand E. et al.which have shown thatinhalation of lipopolysaccharide (LPS) in human volunteers induced activation of bronchial epithelium by increased expression of p38 MAPK kinase and IL-8 (Roos-Engstrand E et al., 2005).

In this paper we can therefore confirm that the epithelium plays a fundamental role in determining the onset of neutrophilic inflammation typical of COPD: the recognition of LPS is an event triggering the activation of TLR4 present on the membrane of the bronchial epithelial cells. In turn, the TLR4, by the Myd88-dependent signal pathway, leads to the activation of p38α MAPK resulting in the transcription of inflammatory proteins, including IL-8.

In this thesis we have also paid attention to the possible role, of human HSP60, as bronchial epithelial stimulating agent,in order to assess itsextracellular behavior.It is now known that HSPs are prototypical DAMPs (damage-associated molecular patterns) mainly derived from the cytoplasm (Pouwels S D et al., 2014) and now called chaperokines (Asea, A et al., 2000), butnot much is known about the ability to perform their autocrine and paracrine action.Some authors have explained that HSPs are able to bindspecific membrane receptors, such as TLR2 and TLR4, andactivate their signaling in a MyD88-dependent manner.This leads to the initiation of the NF-κB and interferon regulatory factor signaling pathways, which ultimately leads to the release of pro-inflammatory cytokines (e.g., tumor necrosis factor, interferons, IL-1β, IL-6, and IL-8) (Asea, A et al., 2000; Tolle, L.B. &Standiford, T.J, 2013).It is still not clear if HSPs have either stimulatory or inhibitory effects on the immune response. It therefore seems that their effects depend on the context in which they are released in extracellular milieu and encountered by the cellular immune response network (Pockley et al; 2007). Today remains not fully elucidated the role of HSP60in COPD, and its action on human

bronchial epithelial cells.In the past few years several studies have been performed to investigate the role of HSPs in COPD and in vitro models, but no one has, to our knowledge, been examined in any detail the HSP60behavior.For these reasons, we investigated in the bronchial mucosa the presence and levels of HSP60. Our "ex vivo" study on bronchial biopsies shows that in the bronchial epithelium and lamina propria HSP60 proteins were significantly increased in severe COPD compared to control smokers and non-smoking subjects. The increased presence of HSP60 in the epithelium could be easily correlated to its cytoprotective function but the presence in lamina propria is suggestive for its intervention in the inflammatory process (Cappello F. et al., 2011). These "ex vivo" results forced us to perform a set of in vitro experimentsto determine whether an oxidant and pro-inflammatory stress(e.g., that caused by cell exposure to H_2O_2) may induce HSP60 up-regulationand release by bronchial epithelial cells (cell line16-HBE). This hypothesisis is in agreement to some authors reporting that oxidative stress, a hallmark of COPD (Ricciardolo FL. Et al., 2005), is a potent HSP inducer, and HSPs may protect cells from death caused by oxidative stress (Arrigo AP, 1998; Takuma K. et al., 2002). Our experiments with a bronchial-epithelial cell line (16-HBE) exposed to a pro-inflammatory stressor (oxidative stress H_2O_2) showed that HSP60 expression increases during stress and is accompanied by HSP60 release into the extracellular medium. The HSP60 increase is due, at least in part, to overexpression of the chaperonin gene, and in this activity the up-regulation of NF-kB-p65 most likely plays a determinant role, as indicated by its capacity to bind the chaperonin-gene promoter region (Cappello F. et al. 2011). This data are in agreement with those reported above where the transcription factor, NF-kB p65 subunit, is overexpressed in bronchial biopsies of COPD patients compared with control non-smokers.

The results described above have opened new avenues for investigation.For this reason the principal aim of this thesis was to investigate how secreted HSP60 may effectively causes pro- or anti-inflammatory effects on bronchial epithelial cells, through an"in vitro"study model.Stimulating the bronchial epithelium with human low endotoxin HSP60, we found a down-regulation of IL-8 mRNA and an up-regulation of IL-10 mRNA after 2h from the start of the stimulus. These data suggest a potential cytoprotective effect of human HSP60 when present in extracellular environment, mediated by IL-10. Western blot analysis confirmed that there is no change of the IL-8 intracellular protein levels between treated and untreated cells. However, Western blot analysis was not very sensitive for the detection of IL-10 protein because IL-10 in our

experiments was under the detection limit. These data have been reported here for the first time for human HSP60 and are in contrast with some authors who have used the bacterial HSP60 as a stimulus. These studies showed that prokaryotic HSP60 has a pro-inflammatory effect,activating the production of pro-inflammatory cytokinesin epithelial cells (Zhang L et al., 2004).Vabulas et al. (2001) and Sasu et al. (2001)demonstrating that the abundance of chlamydial HSP60 (cHSP60) during chronic infection could promote the activation of T and B cells, engaging Toll-like receptors (TLRs) with the induction of cellular signalling networks. The inflammatory effects are mediated through the innate immune receptor complex TLR4-MD2 and proceeds via the MyD88-dependent signaling pathway (Bulut Y et al., 2002). In particular, cHSP60 can stimulate tumor necrosis factor-alpha (TNF-alpha) secretion by macrophages (Kol A. et al., 1998; Bulut Y et al., 2002) and can stimulate endothelial cells, smooth muscle cells and macrophages to produce pro-inflammatory cytokines (interleukin (IL)-11, IL-8, IL-12, IL-6), by activation of the nuclear factor NF-kB (Kol A. et al., 1999; Bulut Y et al., 2002).

To demonstrate the anti-inflammatory effect of human HSP60 we combined pro-inflammatory stimuli such as H_2O_2, LPS and cytomix with human low endotoxin HSP60, respectively. We obtained a total inhibitory effect of HSP60 when combined with the oxidative stimulus (H_2O_2), inhibiting the production of IL-8 mRNA. Combining HSP60 with LPS stimuli, the inhibitory effect of HSP60 was only partial: in fact, after 2h from stimulation, LPS induced the transcription of IL-8 mRNA, eventhough in a minor extent as compared to LPS stimulation alone, but the inhibitory effect of HSP60 was total after 4h from the combined treatment. This partial inhibition may be due to the higher affinity of LPS to bind TLR4 in respect to HSP60. However, combining cytomix with HSP60, any protective effect has occurred.Cytomix is a strong pro-inflammatory stimulus, and the action of HSP60 is not able to limit its effects. This may be due to the presence of different specific receptors on the cell membrane with binding affinity for TNFα, IL-1βand INFγnot interacting with the HSP60 receptor(TLR4).These findings suggest thatHSP60 is a paradoxical molecule withbeneficial effects in the lung:predominantlycytoprotective in intracellular environment and potentially anti-inflammatory when secreted outside cells, and capable of modulating the secretion of pro- and anti-inflammatory cytokines.

Our “in vitro” experiments show for the first timein 16HBE cellsthat human HSP60 interacts with TLR4 and plays critical roles in IL-10 mRNA up-regulation and IL-8

down-regulation, as previously reported. To better understand how extracellular HSP60 induces the activation of the pathway after binding TLR4 we also investigated the mRNA levels of Myd88 (principal TLR4adapter)and the main MAP-kinase involved. Myd88 mRNA was up-regulated after 1h of stimulation with HSP60, this confirms the activation of TLR4 via the MyD88-dependent signaling pathway.Treated cells (16HBE) with HSP60 showed also an up-regulation of p38 mRNA and of CREB1 mRNA transcription factor (phosphorylated by p38 MAPK activation), compared with untreated cells.JNK1 and ERK1 mRNA levels were not changed. These results suggest that p38 MAPK and CREB1 play a more significant rolein inducing IL-8 and IL-10 mRNA increase than ERK1 and JNK1 in bronchial epithelial cells after HSP60 stimulation. These findings are in part in contrast with Zhang et al. (2004) showing that HSP60 activates ERK1/2in epithelial cell. To confirm our data, pre-treatment of HSP60 exposed 16HBE cells with SB203580 (a p38α MAPK inhibitor), abolished the CREB1 and IL-10 mRNA up-regulation and the IL-8 mRNA down-regulation, confirming the involvement of p-38 MAP-kinase and CREB1 in this process. We suppose that HSP60 induces the activation of CREB1, which is known to be important for transcriptional regulation of IL-10 expression (Brenner et al., 2003; Hu et al., 2006). Notably, activation of CREB1 has also been demonstrated to suppress the transcriptional activity of NF-kB by competing with the p65 NF-kB subunit for the transcriptional co-factor CBP (Parry et al., 1997). However, our data have shown that the stimulation with human HSP60 is able to up-regulate IL-10 mRNA through the transcription factor CREB1, modulating the anti-inflammatory activity on the 16-HBE. However, CREB1 is not able to suppress the activity of p65 NF-kB subunit (found increased in treated cells). This is also confirmed in patients with stable COPD where NF-kBis increased compared with control non-smokers (Di Stefano, 2002). We hypothesize that activation of p65 NF-kB is only partially dependent from HSP60 stimulation and that different parallel mechanisms, possibly involving other pro-inflammatory stimuli, using different molecular patways, play a role in this inflammatory process. In conclusion, with this thesis, we investigated the role of HSP60 in both in “ex vivo” and “in vitro” models in order to improve our understanding onthe pro- or anti-inflammatory effect of this important chaperonin. The obtaineddata showed that in bronchial epithelium HSP60 is increased in patients with stable COPD compared with control non-smokers. In addition, in our in vitro model we observed for the first time that HSP60 released into the extracellular environment has an anti-inflammatory activity. HSP60 does not simply

suppressesthe cytokine expression in 16HBE but rather, shifts the cytokine pattern expressed toward an anti-inflammatory response (by CREB1 activation), characterized by suppression of inflammatory cytokines (IL-8 mRNA) andincrease of IL-10 mRNA expression.This study opens the road to investigate and to develop therapeutic means in which chaperones would be central players, as cytoprotective agents. In addition our present results may contribute to demonstrate thatbronchial epithelium plays a fundamental role in determining the onset of neutrophilic inflammation typical of COPD. Phospho-p38-MAPK was certainly the most expressed MAP-kinase after oxidative and bacterial (LPS) challenges, performed in 16HBE cells, with a significantprominent increase of p-38 MAPKα mRNA after stimuli. However, the recognition of LPS is an event triggering the activation of TLR4 present on the membrane of the bronchial epithelial cells. The TLR4by Myd88-dependent signaling pathway leads to activation of p38α MAPK resulting in the transcription of inflammatory proteins, including IL-8.The modulation of these factors related to the anti-bacterial response that develops in COPD, involving p38 MAPK, TLR4 and HSP60, may therefore represent a target in modulating the inflammatory response in patients with COPD and could represent a model for diseases showing similar molecular patterns with a Th1 and neutrophilic prevalent inflammatory responses.

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