# EFFECTS OF CIGARETTE SMOKE ON KILLER CELL ACTIVATION IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

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## Dedication

This thesis is dedicated to my parents, Yadong Wang and Liqin He, my supervisers, Dr. Lucy Fairclough and Dr. Ian Todd, Dr. Jonathan Corne, and my son, Hanze (Brian) Li who give me continuous strength to face up to all challenges throughout my life, without which, none of this would have been possible. I am forever indebted.

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# Abbreviations

| | |
|---|---|
| AAT | $\alpha_1$-antitrypsin |
| ADCC | antibody-dependent cellular cytotoxicity |
| APCs | antigen-presenting cells |
| ATS | American thoracic society |
| BAL | bronchoalveolar lavage |
| BLyS | TNF-related ligand B-lymphocyte stimulator |
| BODE | body-mass index (B), the degree of airflow obstruction (O), dyspnoea (D), and exercise capacity (E) |
| BSA | bovine serum albumin |
| CCR | C-C chemokine receptor |
| COPD | chronic obstructive pulmonary disease |
| CSE | cigarette smoke extract |
| cuS-COPD | current smokers with COPD |
| DCs | dendritic cells |
| DTT | 2,3-dihydroxybutane-1,4-dithiol (dithiotheritol) |
| ECD | phycoerythrin-Texas Red-x |
| ECP | eosinophil cationic protein |
| EDTA | ethylenediaminetetraacetic acid |
| EGF | epidermal growth factor |

| | |
|---|---|
| Egr-1 | early growth response gene 1 |
| ENA-78 | epithelial neutrophils activating protein of 78 kDa |
| EPO | eosinophil peroxidase |
| ET-1 | endothelin-1 |
| exS-COPD | ex-smokers with COPD |
| FACS | fluorescent activated cell sorting |
| Fas-L | Fas ligand |
| FCS | Foetal calf serum |
| $FEV_1$ | forced expiratory volume in one second |
| FGF | fibroblast growth factors |
| FITC | fluorescein isothiocyanate |
| FMO | fluorescence minus one |
| FVC | forced vital capacity |
| $\alpha$-Galcer | $\alpha$-galactosylceramide |
| G-CSF | granulocyte colony-stimulating factor |
| GM-CSF | granulocyte-macrophage colony stimulating factor |
| GOLD | global initiative for chronic obstructive lung disease |
| GPCRs | G-protein-coupled receptors |
| GRO-$\alpha$ | growth-related oncogene-$\alpha$ |
| 4-HNE | 4-hydroxy-2-nonenal-modified |
| HNS | healthy non-smokers |

| | |
|---|---|
| HS | current healthy smokers |
| ICAM-1 | intercellular adhesion molecule 1 |
| iNKT | invariant natural killer T cell |
| iNOS | inducible NO synthase |
| IP-10 | interferon-inducible protein-10 |
| ITAM | immunoreceptor tyrosine-based activation motif |
| ITIM | immunotyrosine-based inhibitory motif |
| KIRs | killer cell immunoglobulin-like receptors |
| LCs | Langerhans cells |
| LFA-1 | lymphocyte function-associated antigen 1 |
| LILRs | leukocyte immunoglobulin (Ig)-like receptors |
| LTB4 | leukotriene B4 |
| LPS | lipopolysaccharides |
| MAC | macrophage antigen |
| MAPK | mitogen-activated protein kinase |
| $MC_T$ | mucosal mast cells |
| $MC_{TC}$ | connective tissue mast cells |
| mDCs | myeloid dendritic cells |
| MHC | major histocompatibility complex |
| MIG | monokine induced by interferon-gamma |
| MMP | matrix metalloproteinase |

| | |
|---|---|
| MMPS | matrix metalloprotease enzymes |
| MPO | myeloperoxidase |
| MRC | Medical Research Council |
| NE | neutrophil elastase |
| NF-κB | nuclear factor-κB |
| NK | natural killer |
| NKT | natural killer T cell |
| NO | nitric oxide |
| PBMCs | peripheral blood mononuclear cell |
| PBS | phosphate buffered saline |
| PC5 | phycoerythrin-cyanin 5.1 |
| PC7 | phycoerythrin-cyanin 7 |
| pDCs | plasmacytoid dendritic cells |
| PE | phycoerythrin |
| $PGE_2$ | prostaglandin $E_2$ |
| $PGF_{2\alpha}$ | prostaglandin $F_{2\alpha}$ |
| RANTES | released by activated normal T cells expressed and secreted |
| REC | Research Ethics Committee |
| ROS | reactive oxygen species |
| RPMI 1640 | Roswell Park Memorial Institute media |

| | |
|---|---|
| RV | rhinovirus |
| TARC | thymus- and activation-dependent chemokine |
| TB | tuberculosis |
| Tc | cytotoxic T cell |
| TCR | T cell receptor |
| TEER | trans-epithelial electric resistance |
| TGF-β | transforming growth factor-β |
| Th | helper T cell |
| TIMPs | tissue inhibitors of MMPs |
| TLA-1 | T-cell intra-cytoplasmic antigen-1 |
| TNF | tumour-necrosis factor |
| Treg | regulatory T cells |
| TSLP | thymic stromal lymphopoietin |
| VEGF | vascular endothelial growth factor |
| WHO | World Health Organization |

## Publications Arising from this Work

1. **J Wang**, Richard A Urbanowicz, Patrick J. Tighe, Ian Todd, Jonathan M. Corne, and Lucy C. Fairclough. Differential Activation of Killer Cells in the Circulation and the Lung: A Study of Current Smoking Status and Chronic Obstructive Pulmonary Disease (COPD). PLoS ONE. 2013 Mar; 8(3): e58556.

2. **J Wang**, Ian Todd, Jonathan M. Corne, Patrick J. Tighe, and Lucy C. Fairclough. Cigarette smoke extract suppresses NK cells activation in current smokers with COPD. Eur Respir J. 2014; in preparation

## Poster Presentations Arising from this Work

1. **J Wang,** Richard A Urbanowicz, Jonathan M. Corne, Ian Todd, and Lucy C. Fairclough. Cytotoxic Cells in Bronchoalveolar Lavage (BAL) Fluid from COPD patients. ATS conference 2009; San Diego, USA.

# Abstract

Chronic Obstructive Pulmonary Disease (COPD) is a chronic inflammatory disease involving both innate and adaptive immune responses. Abnormal numbers of inflammatory cells have been examined in COPD subjects, as well as the effects of cigarette smoking on immune cells and molecules. Killer cells, including $CD8^+$ T cells, NKT-like cells and NK cells, are thought to play a role in the development of COPD through their cytotoxic functions. In this project, we report *ex vivo*, activation levels of these cell types in COPD patients, as well as effects induced by cigarette smoke extract *in vitro*.

PBMCs were collected from healthy non-smokers (HNS), current healthy smokers (HS), current smokers with COPD (cuS-COPD) and ex-smokers with COPD (exS-COPD). Activation levels of interest and CSE effects on them were analysed by flow cytometry.

Killer cells, including $CD8^+$ T cells, NKT-like cells and NK cells, were significantly activated in current smokers with or without COPD compared to healthy non-smokers. Furthermore, KIR (CD158e1) expression was dramatically lower in smokers with or without COPD in comparison with healthy non-smokers. The cytotoxicity of $CD8^+$ T cells from both current smokers and ex-smokers with COPD patients were significantly less than that in healthy volunteers. Also, *in vitro,* CSE markedly decreased

IL-15 treated NK cell activation in current smokers with COPD compared to other three groups. The expression of granzyme B was also significantly inhibited on IL-15 stimulated NK cells when CSE was added.

We conclude systemic *ex vivo* killer cell activation is smoking rather than disease related. Cigrette smoking has immunosuppressive effects on killer cell activation and granzyme B expression in PBMCs from current smokers with COPD.

# Introduction

## 1.1 Chronic Obstructive Pulmonary Disease

### 1.1.1 Definition, Prevalence and Global burden

COPD, as a common, preventable and treatable disease, is characterised as a chronic inflammatory disease causing airway limitation that is usually progressive but not completely reversible (Celli and MacNee, 2004). It is the fourth leading worldwide cause of morbidity and mortality, and is predicted to become the third most frequent burden of disease in the world by 2030, after heart disease and stroke (Rabe et al., 2007). The estimated death rate of COPD shows geographical differences. In the US, according to updated statistics from the Centre for Disease Control (CDC), 0.04% of people died from COPD in 2009. Compared with the decreasing mortality in some diseases, such as heart disease and stroke, COPD is the only major disease with an increasing death rate. In European countries, mortality from COPD varies from 0.025% to 0.075% of the adult population. In Japan, the reported mortality from COPD is 0.04%, whereas nearly 1.3% of deaths result from COPD in China(Buist et al., 2007, Fukuchi et al., 2004, Halbert et al., 2006). Gender differences are also seen: morbidity from COPD is higher in men than in women in the UK (Soriano et al., 2000). Nevertheless, some studies indicate that the prevalence of COPD seems almost equal in men and women and that women are more susceptible to

COPD than men (Xu et al., 1994, Silverman et al., 2000). Medical costs of COPD are an economic burden in both developed countries and developing countries (Chapman et al., 2006). There is a positive correlation between the severity of COPD and the cost of care (Jansson et al., 2002), although costs per patient for COPD are variable across countries due to how health care is provided and paid .

### 1.1.2 Risk factors

Prevalence estimates of the disorder show considerable variability across populations, suggesting that risk factors can affect populations differently. Although only 15% to 20% of smokers develop clinical COPD (Brown et al., 1994), cigarette smoking is still the common and key pathological driver in the development and progression of COPD (Burrows et al., 1977). The Fletcher curve (Figure 1.1) shows the importance of smoking cession in patients with COPD.

Besides, other factors are involved. Genetic predisposition now also seems to play an important role in the development of COPD. $\alpha_1$-antitrypsin (AAT), a major circulating inhibitor of serine proteases, is the best established antiprotese associated with COPD (Hill et al., 1999, Ishii et al., 2000, Sandhaus, 2009, Zielinski and Kuca, 2004). It has been

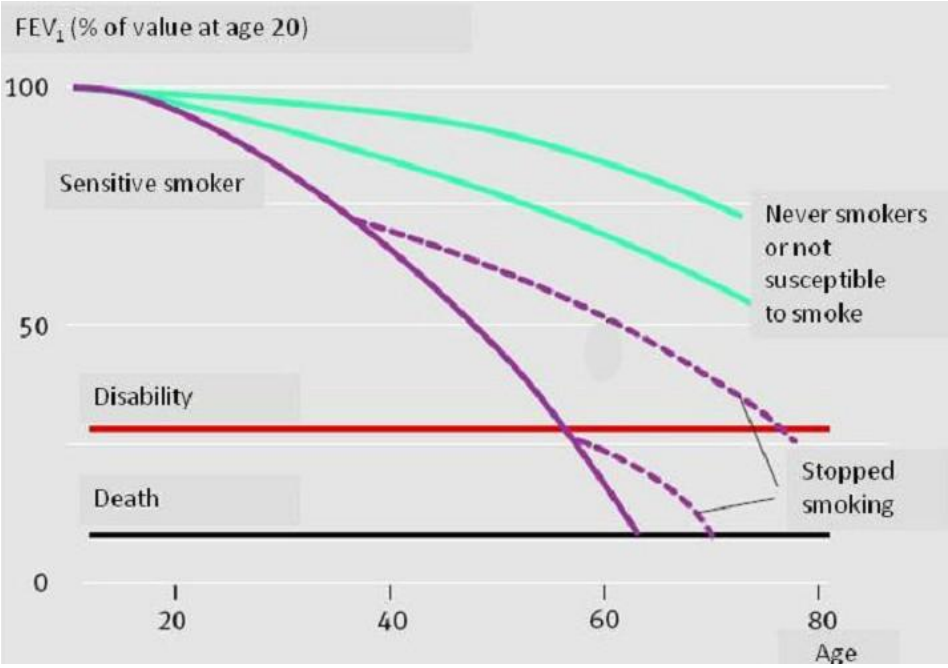



**Figure 1.1 Age-related decline in $FEV_1$ with different smoking history.** (Adapted from Fletcher et al. Definitions of emphysema, chronic bronchitis, asthma, and airflow obstruction: 25 years on from the Ciba symposium. Thorax 1984; 39: 81-85)

observed that AAT deficiency can lead to early onset emphysema in COPD patients (Ekeowa et al., 2009, Eriksson, 1965, Ranes and Stoller, 2005, Strange, 2013). Metalloproteases, proinflammatory cytokines, various antioxidant enzymes and detoxifying enzymes are also linked to COPD (Wood and Stockley, 2006). Occupational exposure to biomass smoke, environmental pollution with noxious particles and gases, chronic asthma, history of pulmonary tuberculosis and poor socioeconomic status can all result in COPD (Balmes et al., 2003, Barker et al., 1991, Ezzati, 2005,

Orozco-Levi et al., 2006, Prescott and Vestbo, 1999, Viegi and Di Pede, 2002). These may explain why there are around one-third of COPD patients who have never smoked (Salvi and Barnes, 2009).

*Cigarette Smoke*

Cigarette smoking has been implicated as an etiological risk factor resulting in various chronic diseases with high morbidity and mortality, including cancers, heart diseases, variety of infections and chronic lung diseases (Almirall et al., 1999, Arcavi and Benowitz, 2004, Sopori, 2002).

The smoke from a cigarette contains thousands of chemicals which have various toxic, mutagenic and carcinogenic activities (Hoffmann and Wynder, 1986, Stedman, 1968). The concentrations of chemical ingredients also vary widely in the different cigarette brands. Studies show that various components of cigarette smoke can affect the immune response in different cell types (Lee et al., 2007b, McCue et al., 2000, Ouyang et al., 2000). Two major components of smoke that leads to many of deleterious effects are nicotine and tar (Hoffmann and Wynder, 1986, Stedman, 1968). Holt *et al* has shown that cigarette smoke containing high levels of tar and nicotine is more immunosuppressive than the smoke

from low-tar low-nicotine cigarettes (Holt et al., 1976). However, effects of cigarette smoke on the immune system are biphasic, which is mainly determined by the components and duration of exposure. For example, unlike nicotine and tar, tobacco glycoprotein and metals present in cigarette smoke are generally immunostimulatory (Brooks et al., 1990, Francus et al., 1992).

Previous studies have demonstrated that cigarette smoke causes structural and functional changes in the respiratory ciliary epithelium (Cantin et al., 2006, Mio et al., 1997, Wyatt et al., 1999), lung surfactant and immune cells (Mohan, 2002a). In COPD patients, abnormal cell numbers of different cell types have been observed, including $CD8^+$ T cells, alveolar macrophages, dendritic cells (DCs), neutrophils and NK cells in bronchoalveolar lavage (BAL) fluid, epithelium and adventitia of small airways, sputum or peripheral blood samples (O'Shaughnessy et al., 1997, Saetta et al., 1998, Baraldo et al., 2004, Barnes, 2004b, Demedts et al., 2007, Urbanowicz et al., 2009). Therefore, accumulating data suggested that chronic inhalation of cigarette smoke alters a wide range of immunologic function, including both innate and adaptive immune responses.

### 1.1.3 Systemic effects of COPD

Studies have shown that COPD is associated with both pulmonary and systemic inflammation. Weight loss, skeletal muscle dysfunction, cardiovascular disease, cancer, nutritional abnormalities and other organ effects all contribute the high burden of COPD together with respiratory symptoms protein (Agusti, 2007, Gosker et al., 2000, Pryor and Stone, 1993, Schols et al., 1996, Skillrud et al., 1986, Van den Heuvel et al., 1999), which result in a pronounced deterioration in health status of life.

### 1.1.4 Comparison of COPD with asthma and tuberculosis

COPD, asthma and tuberculosis (TB) are three main public health problems related to lung worldwide. Studies have shown that these diseases have common risk factors, such as cigarette smoking, low socioeconomic status and dysregulation of host defence functions. Also, they can co-exist, which result in co-morbid conditons. Table 1.1 lists the common charasteristics and differences of among these diseases.

### 1.1.5 Pathology of COPD

Chronic obstructive bronchiolitis (swelling and inflammation of the lining of the airways that results in narrowing and

**Table 1.1 Comparison of COPD with asthma and TB**

| COPD | Asthma | TB |
|---|---|---|
| **Common Features** | | |
| • Pulmonary diseases<br>• All carry a significant burden in terms of morbidity and mortality worldwide<br>• Induce inflammatory responses | | |
| **Differences** | | |
| ***Epidemiology*** | | |
| Worldwide prevalence; Not infectious | High prevalence in the developed countries; Not infectious | Over 95% of TB cases in developing countries; Infectious |
| ***Patients group*** | | |
| Mainly adults ≥40 years old | Mainly young children | Mainly young adults |
| ***Symptoms*** | | |
| Chronic cough ; Dyspnea; Large amounts of mucus production; Wheezing; Chest tightness | Cough (specially at night); Wheezing; Shortness of breath; Chest tightness, pain or pressure; Fatigue | Cough (usually cough up mucus); Coughing up blood; Fatigue; Fever; Excessive sweating, especially at night; Unintentional weight loss |
| ***Burden of mortality*** | | |
| The fourth leading worldwide cause of morbidity and mortality | Most cases are managed at a primary care level. | Second only to HIV/AIDS as the greatest infectious killer worldwide |
| ***Disease development*** | | |
| Airway limitation is not reversible; Have features of asthma | Airway limitation is reversible; Co-exist with COPD | Not all patients develop airway limitation; COPD as a sequel to active TB |
| ***Immune cells mainly involved*** | | |
| Neutrophils, macrophages, $CD8^+$ T cells, NK cells and NKT cells; Both Th1 and Th2 cell-induced immunity | Eosinophils, $CD4^+$ T cells; Mainly Th1 cells-mediated immunity | $CD4^+$ T cells play a crucial role; Mainly Th1 cell-induced immunity |
| ***References*** | | |
| (Sciurba, 2004) | (Braman, 2006).. | (Chakrabarti et al., 2007) |

obstruction of the airways), emphysema (permanent destructive enlargement of airspaces distal to the terminal bronchioles, caused by loss of lung elastic recoil) and mucus hypersecretion (productively coughing and bringing up sputum for >3 months for at least two consecutive years) are recognized as three distinct pathological features of most COPD patients (Barnes, 2000, Kim et al., 1991). They affect all parts of the lungs including parenchyma and airways and increase the resistance of the conducting airways and lung compliance. The severity of COPD is related to the remodelling of the small airways, especially of the terminal bronchioles including increased airway smooth muscle and goblet cell hyperplasia, submucosal gland hypertrophy and epithelial metaplasia (Hogg et al., 2004).

### 1.1.6 Classification of COPD

Currently, two staging systems have been applied to COPD. Global Initiative for Chronic Obstructive Lung Disease (GOLD) has confirmed the classification of COPD with spirometry. According to the two values of the postbronchodilator spirometric measurement, namely, forced expiratory volume in one second ($FEV_1$) and forced vital capacity (FVC), COPD is classified from stage 0 to stage III (Table 1.2) (www.goldcopd.org., 2011). However, COPD patients carry

**Table 1.2 GOLD classification of COPD**

| Stage | Spirometry | Symptoms |
|---|---|---|
| **0: At risk** | Normal spirometry | Chronic symptoms (cough, sputum production) |
| **I: Mild COPD** | $FEV_1/FVC<70\%$<br>$FEV_1$ greater than or equal to 80% predicted | With or without chronic symptoms (cough, sputum production) |
| **II:Moderate COPD** | $FEV_1/FVC<70\%$<br>$FEV_1$ greater than or equal to 30% to 80% predicted<br>IIa: $FEV_1$ greater than or equal to 50% to 80% predicted<br>IIb: $FEV_1$ greater than or equal to 30% to 50% predicted | With or without chronic symptoms (cough, sputum production, dyspnea) |
| **III: Severe COPD** | $FEV_1/FVC<70\%$<br>$FEV_1<30\%$ predicted or $FEV_1<50\%$ plus respiratory failure or clinical signs of right heart failure | |
| Respiratory failure: arterial partial pressure of oxygen<60mm Hg with or without arterial partial pressure of carbon dioxide greater than or equal to 50mm Hg while breathing air at sea level | | |
| ***Source:*** *The GOLD Workshop level* | | |

both physical impairment and mental burden related to their disease and its symptoms, such as anxiety and depression (Gudmundsson et al., 2005, Wagena et al., 2005); the classification of COPD based on GOLD stages is not sufficient for explaining those mental symptoms (Dahlen and Janson, 2002, Hill et al., 2008, Celli et al., 2004). The BODE index (Body Mass-Index, Airflow Obstruction, Dyspnea and Exercise Capacity) is a multidimensional scoring system for COPD. It comprises an assessment of symptoms, a surrogate of the nutritional state, and exercise capacity together with the spirometric measure of airflow ($FEV_1$) (Celli et al., 2004). It has been shown to be superior in predicting hospitalization and the mortality risk of COPD patients and better correlates to health status (Celli et al., 2004, Ong et al., 2005, Ong et al., 2006). Table 1.3 shows the BODE index classification.

To detect COPD early, the disease should be suspected when a patient has with chronic cough and sputum production and a history of COPD risk factors.

**Table 1.3 BODE index for COPD**

| | BODE index point | | | |
|---|---|---|---|---|
| **Variable** | **0** | **1** | **2** | **3** |
| **$FEV_1$% predicted** | ≥65 | 50-64 | 36-49 | ≤35 |
| **Distance walked in 6 minutes (m)** | ≥349 | 250-349 | 150-249 | ≤149 |
| **MRC dyspnoea scale** | 0-1 | 2 | 3 | 4 |
| **BMI ($Kg/m^2$)** | >21 | | ≤21 | |

**Modified MRC Dyspnoea Scale**

0. Breathless only with strenuous exercise
1. Short of breath when hurrying on the level or walking up a slight hill
2. Slower than most people of the same age on the level because of breathlessness or have to stop for breath when walking atown pace on the level
3. Stop for breath after walking about 100 meters or after a few minutes at own pace on the level
4. Too breathless to leave the house or breathless when dressing

## 1.2 Pathological hallmarks in COPD

In the last decade, there have been many studies performed on bronchial biopsies, lung parenchyma and peripheral blood obtained from COPD patients compared with healthy non-smokers and smokers with normal lung function. They provide new insights on the role of the different inflammatory and structural cells, mediators and signalling pathways, contributing to a better knowledge of the pathogenesis of COPD. Immunological cells, such as neutrophils, macrophages, DCs, T lymphocytes, epithelial cells, NK cells, NKT-like cells and so on, are mostly involved in the abnormal inflammatory response that occurs in COPD. These cells play different roles in producing structural changes in the airways including loss of respiratory bronchioles, alveolar ducts and alveoli, which further lead to smooth muscle hypertrophy, epithelial disruption, fibrosis, mucociliary dysfunction and lung destruction. This section summarizes how these immune cells work in COPD patients.

### 1.2.1 Immune cells in COPD

#### 1.2.1.1 Neutrophils

Neutrophils are the only cell type in mammals permitted to migrate to any part of the body (Nathan, 2006). As one of the front line defensive cells of the immune system, they

contribute to both tissue repair and tissue damage (Henson et al., 2006, Weiss, 1989). Once injury happens, activated neutrophils accumulate to infected tissues and release proteases, such as neutrophil elastase, cathepasin G, matrix metalloproteinases (MMPs), and reactive oxygen intermediate including superoxide, onzone, hydroxyl radical that contribute to tissue damage (Nathan, 2006). On the other hand, neutrophils produce destructive oxidizing agents and attempts at blocking neutrophil function that have resulted in significantly diminished early tissue destruction (Brickson et al., 2003). According to this unique capacity and without the serious side effects of increasing the host's risk of infection (Nathan, 2002), targeting neutrophils can be considered as an anti-inflammatory therapeutic in many diseases. Neutrophils are also life-saving decision-markers that trigger and maintain an immune response by producing molecular signals. Generated signals can activate epithelial cells, macrophages, mast cells, endothelial cells, T cells, B cells, and also neutrophils themselves. For instance, tumour-necrosis factor (TNF) produced by neutrophils drives DC and macrophage differentiation and activation (Bennouna et al., 2003, van Gisbergen et al., 2005). TNF-related ligand B-lymphocyte stimulator (BLyS), also secreted by neutrophils, helps to elicit the proliferation and maturation of B cells (Scapini et al.,

2005). Neutrophil activated pro-chemerin, such as chemerin, can attract both immature DCs and plasmacytoid DCs, and monocytes (Chertov et al., 1997, Wittamer et al., 2005). IL-12 activated neutrophils produce IFN-$\gamma$ that helps to drive T cell differentiation and macrophage activation (Ethuin et al., 2004).

There is a homeostatic feedback loop among neutrophils, macrophages and T cells regarding neutrophil production in the bone marrow. Granulocyte colony-stimulating factor (G-CSF) is a necessary regulator of neutrophil proliferation and production. Its secretion is regulated by IL-17 produced by regulatory T cells. Extravascular macrophages release IL-23 as the major factor governing IL-17 production. However, apoptosis of neutrophils by macrophage ingestion can suppress IL-23 production.

Many acute and chronic lung diseases including asthma, COPD, bronchiectasis, cystic fibrosis, acute bronchitis, and lung cancer are associated with neutrophilic inflammation. There is abundant evidence showing neutrophils as primary effector cells in COPD (Barnes, 2007). Studies on BAL, sputum, bronchial epithelium and lamina proparia indicated that smokers with or without COPD have increased numbers of

activated neutrophils compared with non-smokers (Dragonieri et al., 2009, Janoff et al., 1983, Keatings et al., 1996, Lacoste et al., 1993, Pesci et al., 1998). However, neutrophil numbers are only higher in sputum, BAL and small airways than lung parenchyma large airways (Battaglia et al., 2007), which indicate rapid transit through the airways and parenchyma. Also, the percentage of sputum neutrophils was increased with GOLD stage in COPD patients (Singh et al., 2010).

After maturation in the bone marrow, neutrophils are released into the circulation. When lung inflammation occurs, neutrophils migrate from the pulmonary circulation to postcapillary venules and adhere to endothelial cells in the alveolar wall before passing into the alveolar space (Hogg and Walker, 1995). The recruitment of neutrophils from bronchial circulation to the bronchial submucosa is coordinated by the interaction of adhesion molecules on the surface of neutrophils and proteins released by the bronchial endothelial cells. $B_2$-integrin (CD18) is a neutrophil adhesion molecule that facilitates neutrophil adhesion to endothelium via its endothelial counter receptor intercellular adhesion molecule 1 (ICAM-1) (Diamond et al., 1990, Woolhouse et al., 2005). Increased expression of macrophage antigen (MAC)-1 (CD11b/CD18) has been found in COPD patients (Noguera et

al., 1998). Also, IL-8, produced by neutrophils, monocytes, alveolar macrophages, pulmonary epithelium, smooth muscle airway cells, eosinophils and fibroblasts, can cause rapid mobilization of MAC-1 to the neutrophil surface (Huber et al., 1991). Increased levels of IL-8 have been detected in BAL and sputum of patients with COPD (Donaldson et al., 2009, Pesci et al., 1998). E-selectin, which is essential to slow down the circulating neutrophils to increase neutrophilic uptake, is upregulated on endothelial cells in the airways of COPD patients (Di Stefano et al., 1994).

Trafficking neutrophils into the respiratory tract from postcapillary venules is predominantly regulated by the epithelial cells that form a barrier lining the airway of the lung (Hogg and Walker, 1995). Chemotactic signals attract neutrophils accumulation near mucosal epithelial cells and in the lumen of the airway (Tanino et al., 2002, Traves et al., 2002). These signals including IL-8 (Tanino et al., 2002), TNF-$\alpha$ (Higham et al., 2000), granulocyte-macrophage colony stimulating factor (GM-CSF) (Aaron et al., 2001), leukotriene B4 (LTB4) (Crooks et al., 2000), nuclear factor-$\kappa$B (NF-$\kappa$B) (Di Stefano et al., 2002), and ENA-78 (epithelial neutrophils activating protein of 78 kDa) (Papi et al., 2006b) have all been found increased in COPD airways. Above chemotactic factors

may derive from alveolar macrophages and epithelial cells, however, IL-8 and leukotriene B4 (LTB4) are also produced by neutrophils themselves (Profita et al., 2005). Recently, Blidberg and colleague have shown that chemotaxis of circulating neutrophils towards IL-8 and partly towards LTB4 was increased in both smokers with or without COPD (Blidberg et al., 2012).

In the respiratory tract of COPD patients, recruited neutrophils have been shown to infiltrate the airway epithelium and submucosal glands from the proximal to the distal part (Saetta et al., 1997), causing these cells to act as producers of potent secretagogues that result in mucus hypersecretion and emphysema (Damiano et al., 1986, Nadel, 1991). Also, increased numbers of neutrophils and $CD8^+$ T cells infiltrate the smooth muscle in the peripheral airways of COPD patients compared to smokers and non-smokers, which affects the structure and contractility of airway smooth muscle, contributing to peripheral airways obstruction (Baraldo et al., 2004).

Activated neutrophils produce serine proteases including neutrophil elastase (NE), cathepsin G, proteinase-3, macrophage-derived matrix metalloproteinase (MMP)-8, MMP-

9 and myeloperoxidase (MPO) (Borregaard et al., 2007), which promote the bronchial inflammation, alveolar destruction and also potent mucus stimulants (Ilumets et al., 2008, Weiss, 1989). NE has been demonstrated in emphysematous tissue of COPD patients and there is a correlation between emphysema severity and elastase levels in peripheral blood neutrophils (Damiano et al., 1986, Galdston et al., 1977). Higher concentratioin of NE-specific fibrinogen degradation product A$\alpha$-Val$^{360}$ has been observed in sputum in exacerbations of COPD compared to stable COPD patients (Carter et al., 2012). MMP-9 and MMP-12, which are secreted by macrophages, determine the neutrophils influx (Russell and Ley, 2002). They are also increased in COPD patients (Russell et al., 2002).

Once neutrophils arrive in airways, they generate great amount of $O_2^-$ and reactive oxygen species (ROS), which are also produced by activated neutrophils, eosinophils, macrophages and epithelial cells in the airways of COPD patients (MacNee, 2001b). ROS can damage the epithelium, reduce ciliary beat frequency, stimulate mucus production by goblet cells, and increase the permeability of the bronchial mucosa (Rusznak et al., 1996). All of them lead to airway edema and protein exudation in the airway (Gompertz et al.,

2001). Studies on systemic neutrophils have shown that there is an increase in ROS production in COPD patients compared to healthy smokers and healthy nonsmokers (Gustafsson et al., 2000, Rahman et al., 1996).

Apoptosis of neutrophils is the way to terminate inflammation and further prevent any permanent damage. During apoptosis, neutrophils lose their ability to produce granule contents and undertake phagocytosis (Haslett, 1999). Mucociliary escalator can also remove activated neutrophils if apoptosis is not efficiently performed. However, this removal leads to the production of proteases and chemoattractants that continuously destroy the lung tissue (Nathan, 2006). Increased levels of chemotactic factors, such as IL-8, LTB4 and GM-CSF, have shown their capacities of delaying neutrophils apoptosis in COPD patents, which leads to secondary necrosis and increases inflammation (Haslett, 1999). Also, delayed apoptosis by neutrophils from COPD patients is related to the expression of bak, bcl-xl and mcl-1 mRNA (Zhang et al., 2012). Interestingly, reduced spontaneous apoptosis only occurs at exacerbation but not stable COPD patients (Pletz et al., 2004).

In contrast to T lymphocytes, particularly $CD8^+$ T cells, and macrophages that are predominant inflammatory cells in the lung of patients with mild COPD, neutrophils is more potent in severe COPD patients (Di Stefano et al., 2004). Studies on BAL and sputum from COPD patients indicate that the concentration of neutrophils is positively correlated with airflow limitation (Di Stefano et al., 1998, Stanescu et al., 1996). It has also been found that there are increased numbers of neutrophils in bronchial biopsy specimens and peripheral blood during COPD exacerbations (Papi et al., 2006a, Saetta et al., 1994, Zhu et al., 2001). Furthermore, the percentage of apoptotic blood neutrophils at COPD exacerbations is reduced (Schmidt-Ioanas et al., 2006).

To sum up, neutrophils play an important role in the pathology in COPD, especially at exacerbations. Modulation of neutrophil recruitment and apoptosis in the lungs of patients with COPD may provide potential targets of this disease.

#### 1.2.1.2 Dendritic Cells (DCs)

DCs are potent antigen-presenting cells (APCs) and play a crucial role in the initiation of adaptive immune responses (Banchereau et al., 2000). The first defined pulmonary DCs were Langerhans cells (LCs). They are now recognized by their

immunohistochemical expression of CD1a, langerin (CD207) and by electron microscopy the presence of Birbeck granules (Sertl et al., 1986). Recently, three phenotypically and functionally different pulmonary DC subsets have been classified including type 1 myeloid DCs (mDCs, BDCA1, $CD1c^+$), type 2 myeloid DCs (mDCs BDCA3, $CD141^+$) and plasmacytoid DCs (pDCs, BDCA2, $CD303^+/CD123^+$). Myeloid and plasmacytoid DCs perform different functions in both innate and adaptive immunity. They have functional plasticity to induce one of three T-cell responses depending on the type of stimuli, including Th1, Th2, or regulatory T (Treg) cells (Vermaelen and Pauwels, 2005).

The role of DCs in the pathogenesis of asthma has been well studied (Lambrecht and Hammad, 2003). However, studies on the involvement of DCs in the development of smokers with COPD are fairly rare. *In vitro* human studies indicate that DC maturation, their capacity to secrete cytokines and expression of adhesion molecules are affected by nicotine. *Mahyar and his colleagues* have shown that in a nicotinic environment (200ug/ml), both endocytic and phagocytic abilities of monocyte-derived DCs are reduced. Nicotine has no effects on DC maturation in response to bacterial antigen lipopolysaccharide (LPS). Nevertheless, they secrete

dramatically lower levels of pro-inflammatory cytokines, such as IL-12, IL-10, and TNF-$\alpha$. IL-12 is a principal cytokine that bridges innate and specific immune response by activation of NK and T cells and biases the differentiation of CD4 T cells towards IFN-$\gamma$-producing Th1 cells (Macatonia et al., 1995). Low level production of IL-12 by nicotine treated DCs indicates that in a nicotinic environment, the ability of DCs to prime type 1 T-cell polarization is diminished. Moreover, T cells co-cultured with DCs in the presence of nicotine produced significantly lower levels of IFN-$\gamma$ than in the absence of nicotine (Nouri-Shirazi and Guinet, 2003). Another study performed by A*lexandra et al* has shown that nicotine increases the DC expression of adhesion molecules such as the $\beta$2-intergrin lymphocyte function-associated antigen 1 (LFA-1, CD11aCD18) and its ligand CD54 (ICAM-1). Furthermore, the expression of HLA-DR and the DC maturation marker CD83 are also increased in the presence of nicotine (Aicher et al., 2003).

*In vivo* human studies, the number of $CD1a^+$ immature DCs were increased in the alveoli and in the BAL of smokers compared to non-smokers (Casolaro et al., 1988, Soler et al., 1989). However, unlike the airway epithelium that contains exclusive $CD1a^+$ DCs, there are normally relatively few $CD1a^+$

DCs in human alveoli and BAL (Demedts et al., 2005). In COPD patients, the number of langerin ($CD207^+$) immature LCs in the epithelium and the adventitia of small airways is significantly higher compared to never smokers and asymptomatic smokers. This accumulation of DCs is positively correlated to the disease severity. Nevertheless, there is no significant difference in DC numbers in the epithelial layers between current smokers with COPD and ex-smokers with COPD. Also, there is no difference in DC numbers in the lamina propria among groups (Demedts et al., 2007).. Recently, abnormal numbers of DCs subsets were detected in PBMCs from COPD patients. The absolute number of pDCs was significantly reduced along with a marked increase of the mDC/pDC ratio in COPD patients compared to non-smokers (Malta et al., 2013).

CCL20 (also known as MIP3$\alpha$) is a chemokine that attracts DCs toward sites of inflammation via interaction with its receptor CCR6 (Reibman et al., 2003). Its mRNA expression is significantly higher in lung tissue from COPD patients compared to healthy non-smokers and healthy smokers. There is an inverse correlation between the $FEV_1$% and CCL20 mRNA expression. Nevertheless, there is no significant difference in CCR6 mRNA expression in lung tissue between controls and

COPD patients. Similar results have also been found in sputum samples (Demedts et al., 2007). In large airways, current smoking appears to markedly reduce the number of DCs both in the bronchial mucosa and in induced sputum (Reibman et al., 2003). Expression of receptors for antigen recognition, for example, BDCA-1 or Langerin, are significantly increased in mDCs from current smokers with COPD but also markedly decreased in mDCs from ex-smokers with COPD, compared with non-smokers (Stoll et al., 2014).

Overall, investigations on DCs in the pathogenesis of COPD indicate that chronic exposure to cigarette smoking impairs the normal maturation process of DCs and subsequently alters their normal function and interaction with naive lymphocytes, leading to an imbalance of immunity that may increase susceptibility of patients with COPD to respiratory infections.

#### 1.2.1.3 Macrophages

Monocytes, DCs and macrophages, along with neutrophils and mast cells, are all phagocytic cells that are strategically located throughout the body's tissues, where they ingest and process foreign molecules, apoptose cells and debris, maintaining homeostasis, as well as producing multiple mediators to recruit other immune cells (Erwig and Henson,

2008, Mantovani et al., 1972). Macrophages account for the majority of inflammatory cells recovered by airway lavage, regardless of subject status, such as whether or not they are smokers or suffering from airway diseases (Kuschner et al., 1996, Linden et al., 1993). Studies on macrophages have shown that macrophages play a potent role in the pathophysiology of COPD (Barnes, 2004a, Shapiro, 1999).

Numbers of macrophages in BAL fluid, sputum, small airway epithelium, lung parenchyma, bronchial submucosal and bronchial glands are all increased in COPD patients (Barnes, 2004d, Saetta et al., 1993, Saetta et al., 1997, Wallace et al., 1992). In particular, compared to healthy smokers, the number of macrophages is significantly increased by up to 25-fold in the alveolar spaces and tissue in patients with emphysema (Wallace et al., 1992). Also, there is a positive correlation between macrophage numbers in the alveolar walls and the presence of mild-to-moderate emphysema as well as the degree of small airways disease in COPD patients (Di Stefano et al., 1998, Finkelstein et al., 1995, Meshi et al., 2002).

Increased recruitment of monocytes from the circulation in response to monocyte-selective chemokines may be one of

the reasons to account for the increased numbers of macrophages in the lungs of smokers and patients with COPD. Expression of C-C chemokine receptor 2 (CCR2) is thought to be particularly important for macrophage differentiation and recruitment to the airways (Lee et al., 1999), which is 1.4-fold higher levels in macrophages in COPD patients compared to subjects without COPD. Macrophages have a very low proliferation rate in the lungs, however, increased proliferation has been reported to be responsible for the increased numbers of macrophages detected in smokers (de Boer et al., 2000, Barbers et al., 1991, Bitterman et al., 1984). The life span of tissue macropahges ranges from 6 to 16 days. Tissue macrophages are renewed by local proliferation of progenitor cells rather than by monocyte influx into tissue (Sawyer, 1986, Tarling et al., 1987). This feature protects patients from an overwhelming risk of fatal infection when neutrophil production is transiently interrupted. Particles from cigarette smoke can be found within the cytoplasm of alveolar macrophages in smokers for more than two years after smoking cessation (Marques et al., 1997). Therefore, smoking may contribute to the prolonged cellular activation of macrophages. In smokers and patients with COPD, markedly increased expression of the anti-apoptotic protein Bcl-$X_L$ and expression of cyclin-dependent kinase inhibitor $p21^{CIP/WAF1}$ in

the cytoplasm are detected in macrophages (Tomita et al., 2002). These findings suggest that apoptosis of alveolar macrophages may be defective in smokers and in COPD patients, which may be another explanation for the accumulation of macrophages in the lung. In COPD patients and healthy smokers, the expression of apoptosis inhibitor of macrophages in alveolar macrophages is significantly increased in the lung of COPD patients and healthy smokers. Likewise, apoptosis inhibitor of macrophages expression was enhanced in response to CSE exposure (Kojima et al., 2013).

Macrophages tend to display polarized phenotypes by which they can be divided into two major subpopulations, namely, M1 and M2. M1 are classically activated macrophages that exhibit pro-inflammatory properties. These cells play important effector functions including the production of reactive nitrogen species and they secrete high amounts of pro-inflammatory cytokines, such as TNF-$\alpha$, IL-12 and IL-1$\beta$ that regulate antigen presentation to T cells and promote Th1 immunity. In contrast, M2 macrophages have poor antigen presenting capacity but a better clearance of apoptotic cells. They produce anti-inflammatory cytokines, such as IL-10 and TGF-$\beta$. Mannose receptor (CD206) and the scavenger receptor CD163 are two representative markers to distinguish M1 and

M2. *In vitro*, GM-CSF can generate M1 macrophages whereas M-CSF can activate M2 macrophages from human peripheral blood monocytes (Murray and Wynn, 2011, Verreck et al., 2006). In the lung, alveolar macrophages predominantly show anti-inflammatory M2 features that have highly phagocytic capacity (Blumenthal et al., 2001, Van den Heuvel et al., 1999). Figure 1.2 shows the macrophage polarization and their features.

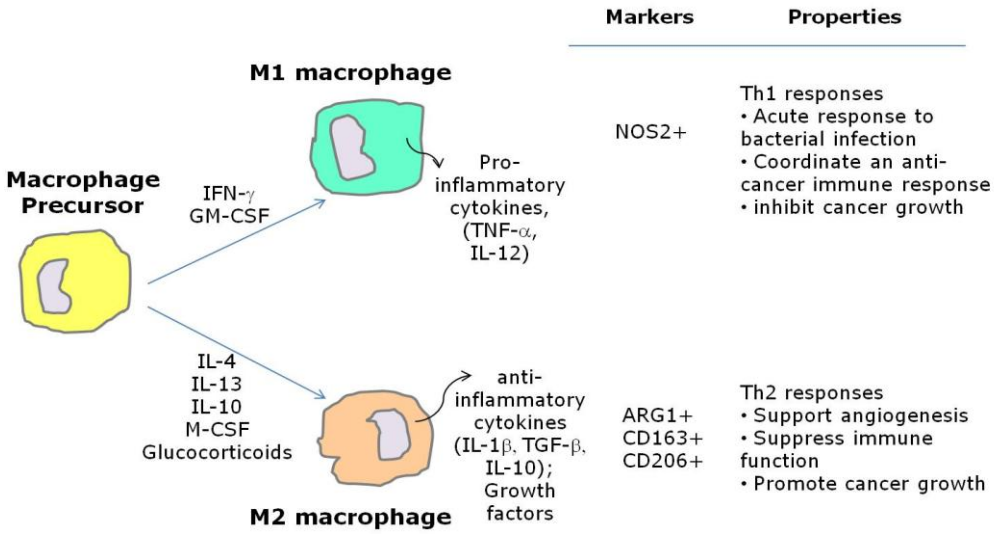



**Figure 1.2 Macrophage polarizations.** Diversity and plasticity are two hallmarks of macrophages. M1 macrophages (classically activated macrophages) are pro-inflammatory and have a central role in host defense against infection, while M2 macrophages (alternatively activated macrophages) are associated with responses to anti-inflammatory reactions and tissue remodeling, and they represent two terminals of the full spectrum of macrophage activation. *Adapted from: M.C. Schmid & J.A. Varner, J Oncol. 2010:201026*

Decreased phagocytosis of alveolar macrophages has been detected in smokers with COPD and smoking cession contributes to improvement (Berenson et al., 2006, Hodge et al., 2007, Taylor et al., 2010). This finding suggests that there may be a regulation of macrophage polarization in COPD patients. Kunz and colleagues recently showed that the percentage of macrophages with M2 characteristics is markedly higher in BAL from ex-smokers with COPD compared to current smoking COPD patients. Also, the percentage of M2 in BAL is higher than in induced sputum, which indicates that the M2 macrophage subpopulation is mainly present in the periphery of the lung. In addition, there is no significant difference in the percentage and number of M2 macrophages in BAL between ex-smokers and current smokers suffering from COPD. Although there are other studies to show the shift of macrophage polarization in COPD (Kunz et al., 2011, Frankenberger et al., 2004, Hogger et al., 1998, Kollert et al., 2009), disease severity may be a possible explanation for these controversial findings. Impaired phagocytosis of alveolar macrophages for *Haemophilus influenzae* and *Moraxella catarrhalis* has been detected to be correlated with the severity of COPD (FEV1% predicted) in COPD patients (Berenson et al., 2013). Moreover, Metcalfe and colleague showed that cigaretter smoke extract significantly reduced

innate immune response of alveolar macrophages from COPD patients (Metcalfe et al., 2014).

Macrophages induce mucus hypersecretion and lung emphysema via production of LTB4, IL-1 and matrix metalloprotease enzymes (MMPS). *In vitro*, alveolar macrophages from COPD patients express increased levels of MMP-1 and MMP-9 (Finlay et al., 1997a, Segura-Valdez et al., 2000), and, in vivo, expression of MMP-2, MMP-9 and MMP-12 are enhanced in COPD patients (Molet et al., 2005, Montano et al., 2004).

#### 1.2.1.4 T cells

T cells in the lung make up a significant proportion of the total T cell population of the body (Pabst and Tschernig, 1995). They are derived from the progenitor cells that begin recombination of four gene segments, namely, $\alpha$ and $\beta$, or $\gamma$ and $\delta$, which build up the T cell receptor (TCR) genes (La Motte-Mohs et al., 2005, Lauritsen et al., 2006). The TCR is composed of two disulfide-linked chains, either $\alpha\beta$ or $\gamma\delta$. TCR-$\alpha/\beta$ cells comprise 95% of the T cell population and regulate the immune response by production of cytokines and chemokines. TCR-$\gamma/\delta$ cells play key functions in mucosal

homeostasis and in response to tissue damage at the epithelial surface (Jameson et al., 2003).

*α/β T cells*

Cytotoxic T cells (Tc cells, $CD8^+$) and helper T cells (Th cells, $CD4^+$) are αβ T cell subsets. Tc cells consist of 30-40% of the total α/β T cell population and recognize intracellular antigens, such as viral or tumour proteins, presented by MHC class I molecules. Th cells constitute 60-70% of the total α/β T cells and recognize extracellular antigens, such as bacterial peptides, presented by MHC class II molecules (Alam and Gorska, 2003, Parkin and Cohen, 2001). The predominant function of Tc cells is to kill infected cells via producing cytokines such as IFN-γ and toxic molecules like perforin and granzymes (Henkart, 1985, Zagury, 1982). Perforin is a pore-forming protein that helps granule proteins of Tc cells to be transported into target cells (Froelich et al., 1996, Shresta et al., 1999). Granzymes and T-cell intra-cytoplasmic antigen-1 (TLA-1) are two main Tc granule proteins that have the capacity to activate caspase mediated DNA fragmentation resulting in apoptosis of the target cells (Kojima et al., 1994). In addition, binding of Fas-ligand presented by Tc cells to Fas expressed by target cells can also induce apoptosis of infected cells (Nagata and Golstein, 1995). Th cells are coordinating

cells of the immune response. They play a major part in activation of B cells.

Tc cells include two subsets, namely, Tc1 cells and Tc2 cells. Tc1 cells produce IFN-γ whereas Tc2 cells release IL-4 and IL-5 (Mosmann et al., 1997). Th cells are also divided into two main groups, namely, Th1 cells and Th2 cells. Th1 subset secretes IL-2 and IFN-γ and is associated with cell-mediated functions, such as activating Tc cells and delayed hypersensitivity. Although both Th1 and Th2 subset can activate B cells and induce antibody production, the main difference is in the profile of isotype switching- Th1 induce mainly IgG1-3; Th2 induce IgG1-4, IgG3 and IgG A (Mosmann et al., 1997).

Besides $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells, Treg cells are a special subpopulation of T cells with the major function of suppressing activation of the immune system by producing suppressor molecules, such as IL-10 or TGF-β (Cederbom et al., 2000, Liu et al., 2003).. $CD4^+$ cells expressing high levels of CD25 (CD25 bright) are thought to be Treg cells that represent between 1 and 3% of total $CD4^+$ T cells (Dejaco et al., 2006). Besides CD4 and CD25, FoxP3 is mostly restricted to $CD4^+$ Treg cells, although some $CD8^+$ T

cells do express it (Fontenot and Rudensky, 2004, Tang and Bluestone, 2008). Recently, CD127 has been identified as a unique cell surface marker for Treg cells (Liu et al., 2006, Shen et al., 2009). In particular, $CD127^{dim}$ has been shown to be a specific marker for Treg cells, whereas $CD127^{bright}$ is expressed by non-regulatory T cells.

*γ/δ T cells*

γ/δ T cells are preferentially associated with epithelial tissue. They are abundant in the gastrointestinal mucosa and skin, which have a potent role in epithelial repair. Unlike α/β T cells, γ/δ T cells do not recognize antigen as peptides presented by MHC molecules. They directly distinguish a great amount of diverse antigens, such as the non-classcial MHC class I molecules T10 and T22, without clonal expression (Hayday and Tigelaar, 2003, Holtmeier and Kabelitz, 2005).

In 1995, Finkelstein *et al* showed an increase of $CD3^+$ T cells in the lung parenchyma in smokers and there was a positive correlation between the number of T cells of the lung tissue and the extent of emphysema (Finkelstein et al., 1995). Further studies have also shown that numbers of $CD4^+$ T cells and $CD8^+$ T cells are both increased in the lung parenchyma and airways in COPD patients compared to asymptomatic

smokers and healthy non-smokers, with a predominance of $CD8^+$ T cells. This is in contrast to the findings in asthma, in which the predominantly increased cells are $CD4^+$ T cells and eosinophils. Abnormal numbers of $CD8^+$ T cells in smokers with COPD is not tissue specific. They are detectable in different tissue compartments, such as large airways sub-epithelium (Lams et al., 2000, O'Shaughnessy et al., 1997), peripheral airway (Saetta et al., 1998), parenchyma (Saetta et al., 1999), smooth muscle (Baraldo et al., 2004), bronchial arteries (Peinado et al., 1999, Saetta et al., 1999, Turato et al., 2002), peripheral blood (Urbanowicz et al., 2009), sputum samples (Urbanowicz et al., 2010), and small airways such as BAL (Saetta et al., 2003, Saetta et al., 2002). Some studies demonstrate the correlation between the number of $CD8^+$ T cells and lung function (Di Stefano et al., 2001, O'Shaughnessy et al., 1997, Turato et al., 2002). However, recently one study demonstrated that the distribution of T-cell subsets in BAL of patients with mild to moderate COPD is smoking *pe se* but not correlated with airway obstruction (Forsslund et al., 2014). Smokers with or without COPD show higher percentages of $CD8^+$ T cells and NKT-like cells than those never smokers and ex-smokers with COPD. Altough toll-like receptors (TLRs) are expressed at low levels in the lung tissue, the percentage of $CD8^+$ T cells expressing TLRs

including TLR1, 2, 4, 6 and 2/1 is significantly elevated in smokers with COPD compared to smokers with normal lung functions (Freeman et al., 2013). Activated $CD8^+$ T cells in COPD secrete Th1 predominant cytokine pattern that includes increased production of IFN-$\gamma$, interferon-inducible protein-10 (IP-10), and monokine induced by interferon-gamma (MIG). In turn, these mediators can cause tissue destruction through the up-regulation of MMP production by macrophages and other immune cells (Grumelli et al., 2004, Maeno et al., 2007). Enzymes released by $CD8^+$ T cells, such as granzymes and perforin, as well as binding of Fas-ligand to Fas between $CD8^+$ T cells and target cells, can also cause cell apoptosis (Henkart, 1994, Kojima et al., 1994).

Although studies of $CD4^+$ T cells are less extensive than $CD8^+$ T cell infiltrates, $CD4^+$ T cells have also been shown to be increased in airways, lung parenchyma and peripheral blood in COPD patients (O'Shaughnessy et al., 1997, Saetta et al., 1999), particularly in proximity to bronchus associated lymphoid tissue (Hogg et al., 2004). Decreased number of $CD4^+$ helper T cells is detectable in BAL from smokers with or without COPD in comparion with non-smoking participants (Forsslund et al., 2014). $CD4^+$ helper T cells are mainly responsible for orchestrating downstream immune process by

producing cytokines. They are essential for the full development of adaptive immune cytotoxicity by priming and promoting the long-term survival of $CD8^+$ T cells. They also help activation and differentiation of antibody elaborating B-cells (Wan, 2010).

Treg cells ($CD4^+CD25^+$ T cells) are a subpopulation of $CD4^+$ T lymphoctyes with significant anti-inflammatory and immunomodulatory effects (Bluestone and Tang, 2005, Jiang and Chess, 2006). Recently, more studies have shown interests in the Treg cells in the pathogenesis of COPD. However, the findings are conflicting. Smyth *et al* demonstrated the up-regulation of Treg cells in BAL fluid in healthy smokers and COPD patients, compared to healthy non-smokers, and there is a positive correlation between FoxP3 expression and the number of pack-years (Smyth et al., 2007). Nevertheless, Barcelo *et al* found increased levels of Treg cells only in BAL from smokers but not in COPD patients and healthy non-smoking participants. Also, the number of Treg cells were higher in BAL than in the peripheral blood in the three groups of individuals studied (Barcelo et al., 2008). Human Treg cells are classifed into three subsets including CD25(++)CD45RA(+) resting Tregs (rTregs), CD25(+++)CD45RA(-) activated Tregs (aTregs), which play

suppressive functions, and CD25(++)CD45RA(-) cytokine secreting (Fr III) cells. Decreased proportions of rTregs and aTregs, and increased proportion of Fγ III cells among $CD4^+$ T cells were found in COPD compared to normal smokers (Hou et al., 2013).

γ/δ T cells play a potent role in tissue repair and mucosal homeostasis. However, only a few studies address the potential role of γ/δ T cells in COPD. Richmond et al showed the number of γ/δ T cells was increased in the bronchial glands of healthy smokers compared to healthy non-smokers (Richmond et al., 1993). Likewise Majo *et al* also found increased γ/δ T cells in the lung parenchyma of healthy smokers (Majo et al., 2001). Recently, two studies both confirmed that the number of γ/δ T cells are also increased in both peripheral blood and BAL fluid from healthy smokers, compared to COPD patients and healthy non-smokers, particularly in BAL (Ekberg-Jansson et al., 2000, Pons et al., 2005). Also, current smokers with normal lung function showed a significant up-regulation of γ/δ T cells compared to ex-smokers with normal lung function (Pons et al., 2005). Importantly, the active smoking inducing up-regulation of γ/δ T cells was blunted in current smokers with COPD, which

indicates the capacity for tissue repair involving γ/δ T cells might be postponed in COPD patients.

Cytokines, chemokines and enzymes producing by T cells perform different roles in cell recruitment, cell activation, cell differentiation as well as killing infected cells. Molecular mechanisms of these molecules in COPD will be demonstrated individually in later sections.

#### 1.2.1.5 NKT cells

NKT cells share receptor structure and function with both T cells and NK cells (Kronenberg and Gapin, 2002). They express T cell membrane proteins, such as CD3, CD4 and CD8, as well as NK cell markers including CD56, CD161 and inhibitory natural killer cell receptors (Emoto and Kaufmann, 2003). The most widely studied subset of NKT cells is invariant NKT (iNKT), which express both an invariant TCR consisting of the α-chain (Vα24/JαQ in humans and Vα14/Jα281 in mice) paired with a β-chain (Vβ11 in human and Vβ2, Vβ7 or Vβ8 in mice) and NK cells markers, such as NK1.1 and Ly-49 (Berzins et al., 2004, Stenstrom et al., 2004). The unique invariant TCR of NKT cells recognizes glycolipid antigens, such as the synthetic glycolipids α-galactosylceramide (α-Galcer) and the endogenous

isoglobotrihexosylceramide (iGb3) by interaction with the MHC class I-like molecule CD1d presented by antigen presenting cells (Burdin and Kronenberg, 1999, Crowe et al., 2003, Zhou et al., 2004). However, recognition of CD1d by NKT cells appears to be tissue specific (Hong et al., 1999). $\alpha$-Galcers has stronger immunostimulatory activities and has been used as a powerful tool for analyzing NKT cell function *in vivo* because conventional T cells do not recognize this molecule (Crowe et al., 2003). There is an overwhelming body of evidence showing that most NKT cells are thymus-dependent, however, some argue for an extrathymic origin (Bendelac, 1995, Bendelac et al., 1997, Crowe et al., 2003, MacDonald, 1995).

In humans, most studies on NKT cells have been limited in peripheral blood. However, one study has shown that NKT cells are clearly detectable in human liver at approximately 4% of hepatic T cells (Doherty et al., 1999). Recently, it has been shown that iNKT cells are found in low numbers in the BAL fluid (less than 2% of total $CD3^+$ T cells), induced-sputum (less than 1.3% of $CD3^+$ T cells) and bronchial biopsy (less than 1.7% of $CD3^+$ T cells) in subjects with mild or moderately severe asthma, subjects with stable or exacerbated COPD and healthy volunteers (Vijayanand et al., 2007). Furthermore,

there are no significant differences among those three groups. The distribution of NKT cells in other human tissues remains to be determined.

According to the expression of CD4 or CD8 surface molecules on NKT cells, they are classified into three subpopulations, namely, $CD4^+CD8^-$ NKT cells (CD4 NKT cells), $CD4^-CD8^+$ NKT cells (CD8 NKT cells) and $CD4^-CD8^-$ NKT cells (DN NKT cells). Recent studies have highlighted the distinct Th1- and Th2-type cytokine profiles of these subsets of NKT cells (Gumperz et al., 2002, Takahashi et al., 2002, Takahashi et al., 2000). Upon stimulation, the CD4 NKT cells produce Th1 cytokines (e.g. IFN-$\gamma$) and Th2 type cytokines (e.g. IL-4), plus IL-2, IL-10 and granulocyte-macrophage colony-stimulating factor (GM-CSF). By contrast, CD8 NKT cells and DN NKT cells predominantly secrete Th1 cytokines (e.g. IFN-$\gamma$) but not IL-2, GM-CSF or Th2 cytokines (Kim et al., 2002, Lee et al., 2002). The different cytokine production of NKT cell subpopulations has different actions on immune effector cells in adaptive immune responses. For example, CD4 NKT cells induce the greatest activation of NK cells, $CD4^+$ T cells and B cells but have no effect on $CD8^+$ T cells. These observed stimulatory activities might be involved in both Th1- and Th2-type immunity. Compared with the other two NKT cell subsets, CD8

NKT cells have substantially greater stimulatory activities on $CD8^+$ T cells while producing less IFN-$\gamma$.

One of the interesting features of the NKT cells is its paradoxical function in different diseases including malignancies and autoimmune dysfunctions. It is hard to conclude which kind of NKT cell subset performs more important roles in immune response. This is dependent on the distribution of NKT cell subsets in different tissues. For example, DN NKT cells are at greater frequency than CD4 NKT cells and CD8 NKT cells in human liver (Kenna et al., 2003). Moreover, activation of NKT cells can be either beneficial or detrimental (Duarte et al., 2004, Kikuchi et al., 2001, Kim et al., 2005, Sharif et al., 2002, Terabe et al., 2000). Previous studies have raised the prospect of manipulating NKT cells for therapeutic benefits (Parekh et al., 2004, Yu et al., 2005). Phase I cancer clinical trials revealed that soluble $\alpha$-GalCer treatment is safe, but exerts moderate immunostimulatory effects (Major et al., 2004, Nakai et al., 2004, Tupin et al., 2004). Depending on differences in cytokine profiles in NKT cell subsets, non-selective activation of all NKT cell subsets could likely result in unexpected immunological outcomes for the intended therapeutic use. This suggests that clinical trials to develop the therapeutic potential of NKT cells should

probably utilize strategies that activate or bias toward specific NKT cell subpopulations rather than the whole NKT cell population. For examples, the numbers of NKT cells were reduced significantly in smokers with COPD compared with non-smoking healthy volunteers and healthy smokers whereas the relative proportion of $CD8^+$ NKT cells was increase in peripheral blood from COPD patients (Urbanowicz et al., 2009).

In addition, cytokine release by NKT cells is always rapid and transient, with a peak of activity within 24 hours. After 24 hours, the production of these cytokines, for example IFN-$\gamma$, quickly returns to baseline levels (Lin et al., 2006). Thus, the timing of activation of NKT cells is also an important factor to achieve therapeutic efficacy.

Besides cytokine production, NKT cells also exhibit potent cytotoxic functions. They can induce cell apoptosis by constitutively expressing Fas-L that interacts with $Fas^+$ target cells (Arase et al., 1994). They also kill tumor targets in a perforin-dependent manner (Smyth et al., 2000).

The capacity of rapidly producing high levels of immunoregulatory cytokines may allow NKT cells to regulate adaptive immunity and autoimmune diseases. Previous studies

have shown that NKT cells are critical in both innate and adaptive immune responses against viral and bacterial infections (Grubor-Bauk et al., 2003, Hansen and Schofield, 2004, Kakimi et al., 2000, Skold and Behar, 2003), as well as in tumour immunity and certain autoimmune and allergic diseases such as type 1 diabetes and allergic asthma (Lisbonne et al., 2003, Smyth et al., 2000).

Recently, Kim *et al* introduced a new mouse model to detect invariant NKT cell function in both asthma and COPD (Kim et al., 2008). They found that, in mouse lung tissue, the chronic pulmonary symptoms required interactions between $CD4^-$ NKT cells and macrophages via CD1d molecules. IL-13, derived from iNKT cells, also served as a source of early anti-inflammatory cytokine with great activity on activation of macrophages. This activation resulted in an autocrine signalling loop due to more IL-13 produced by activated macrophages (Martinez et al., 2009). Similar results were seen in the COPD group. Increased numbers of macrophages expressing high levels of IL-13 were observed in COPD affected lungs removed from transplant recipients compared with normal lungs (Kim et al., 2008).

To date, a number of studies have shown differences of distribution and function of NKT cells in peripheral blood, induced sputum and tissues. For instance, Urbanowicz *et al* have demonstrated remarkable findings on NKT cell features in peripheral blood from COPD patients (Urbanowicz et al., 2009). The numbers of NKT cells were reduced significantly in smokers with COPD compared with non-smoking healthy volunteers and healthy smokers whereas the relative proportion of $CD8^+$ NKT cells was increased. The expression of cytotoxic effector molecules, namely, perforin and granzyme B, were both markedly lower in COPD subjects. In contrast to smoking-free healthy participants and healthy smokers, NKT cells isolated from peripheral blood of COPD patients also showed less cytotoxic activity. Su *et al* further confirmed and extend these findings (Chi et al., 2011). The number of iNKT cells was significantly reduced in peripheral blood from COPD patients compared to healthy smokers. Furthermore, the frequency of iNKT cells is lower in peripheral blood from patients with exacerbations of COPD compared to stable COPD patients. Interestingly, the proportion of NKT cells is dramatically higher in induced sputum from smokers with COPD compared to healthy smokers and healthy non-smokers (Urbanowicz et al., 2010). Also, the expression of both perforin and granzyme B by NKT cells is also increased in

COPD patients compared to the other two groups, which means that NKT cells from COPD patients are more cytotoxic than those from healthy smokers and non-smoking subjects.

NKT cells, as a unique lymphocyte population sharing characteristics with both conventional T cells and NK cells, are involved in immediate immune responses through their efficient cytokine secretion and lytic activity. Therefore, it is necessary to investigate their roles in health and disease. Previous studies on NKT cell performance in COPD highlight that it is important to design clinical studies aiming to modify NKT cell activity for therapeutic benefit.

#### 1.2.1.6 Natural killer (NK) cells

NK cells, as a critical innate immune effector cell type, are an important source of immunoregulatory cytokines and play potent roles in early host defence by interaction with other immune cells. They are equipped with a great number of receptors that can be either inhibitory or stimulatory and also possess certain adhesion molecules that facilitate NK cell homing and trafficking (Gregoire et al., 2007, Lanier, 1998, Lanier et al., 1998). Upon activation, NK cells release a number of cytokines including myeloid-differentiation or activation factors (IL-3, TNF-$\alpha$, GM-CSF), IFN-$\gamma$ and type 2

cytokines (IL-4, IL-5, IL-10 and IL-13) and cytotoxic molecules, such as granzymes and perforin (Cooper et al., 2001a, Cooper et al., 2001b, Loza et al., 2002). These important properties warrant NK cells multiple functions in both innate and adaptive immune system. Killing infected cells by cytotoxic molecules, inducing adaptive immunity through cytokine and chemokine secretion, and providing co-stimulatory molecules to T cells and B cells are main immunological functions of NK cells.

CD56 is an isoform of the human neural cell adhesion molecule and widely used as a cell surface marker to identify NK cells ($CD3^{-}CD56^{+}$). Although its function in NK cells is still unexplored, early studies suggest that CD56 may function as an adhesion protein mediating interactions between NK cells and target cells (Lanier et al., 1989, Nitta et al., 1989, Suzuki et al., 1991). CD16 (Fc$\gamma$RIII), as a receptor of IgG, is also detectable on NK cells (Dall'Ozzo et al., 2004, Lanier et al., 1986). According to the intensity of CD56 and CD16 expression, NK cells have been classified into two subsets including $CD56^{bright}$ and $CD56^{dim}$ NK cells (Cooper et al., 2001a). In peripheral blood, $CD56^{bright}$ NK cells that rarely express CD16 molecule only comprise of approximately 10% of total NK cells and function as cytokine-producing cells with

low level expression of perforin (Konjevic et al., 1995, Nagler et al., 1989). $CD56^{dim}$ NK cells expressing high density of CD16, however, are a majority (~90%) of NK cells and defined as killers due to their high cytotoxicity. The perforin levels of these cells are also high (Nagler et al., 1989). Besides CD56 and CD16, cytokine receptors, such as receptors for IL-2 and IL-15, and other activation or inhibitory receptors are also expressed on NK cells. They can selectively recognize MHC class I ligands, MHC class-I related ligands and host-encoded non-MHC ligands (Lanier, 1998).

Adhesion molecules are thought to play functions in cell-cell interactions, trafficking and homing. Two subpopulations of NK cells have distinct expression patterns of these molecules that result in their functional diversity. In contrast to $CD56^{dim}$ NK cells that express high levels of LFA-1 and PEN5 (a sulfated polylactosamine carbohydrate epitope), $CD56^{bright}$ NK cells highly express CC-chemokine receptor 7 (CCR7) and L-selectin that both are important for homing immune cells to secondary lymphoid organs (Frey et al., 1998), which also may be a reason to explain why $CD56^{bright}$ NK cells are highly presented in lymph node in contrast to $CD56^{dim}$ NK cells (Ferlazzo et al., 2004). Figure 1.3 presents the phenotypes of NK cells with their functional molecules.

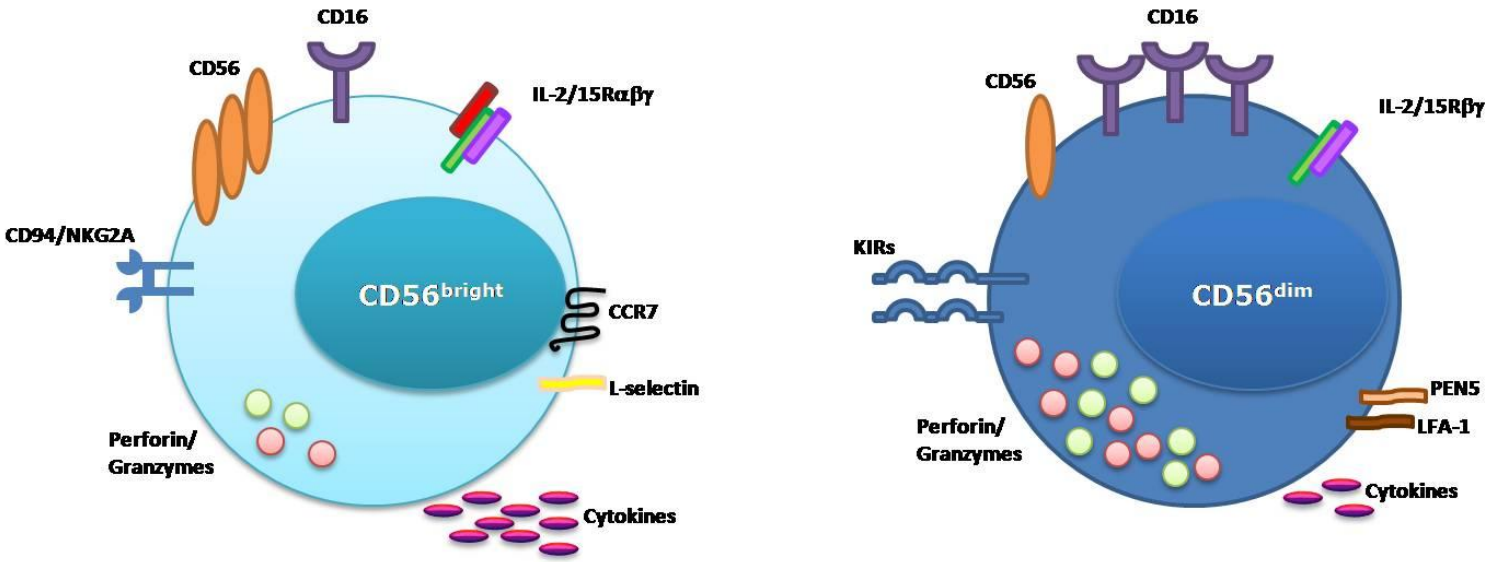



**Figure 1.3 Properties of human NK cell subsets.** $CD56^{bright}$ NK cells (left in the figure) express high levels of inhibitory receptor CD94/NKG2A, cytokine receptors IL-2/15R$\alpha\beta\gamma$ and adhesion molecules including L-selectin and CCR7. However, CD16 and KIRs are rarely expressed on these cells. Upon activation, these cells can release high levels of cytokines. Thus, the predominant function of $CD56^{bright}$ NK cells is immunoregulatory. By contrast, $CD56^{dim}$ NK cells express high levels of CD16 and KIRs. They can secrete a great amount of cytotoxic proteins including perforin and granzyme B but release few cytokines after stimulation. Therefore, they mainly perform as killer cells in the immune system. (Adapted from Megan et al. The biology of human natural kill-cell subsets. 2001. TRENDS in Immunology. 22: 633-640)

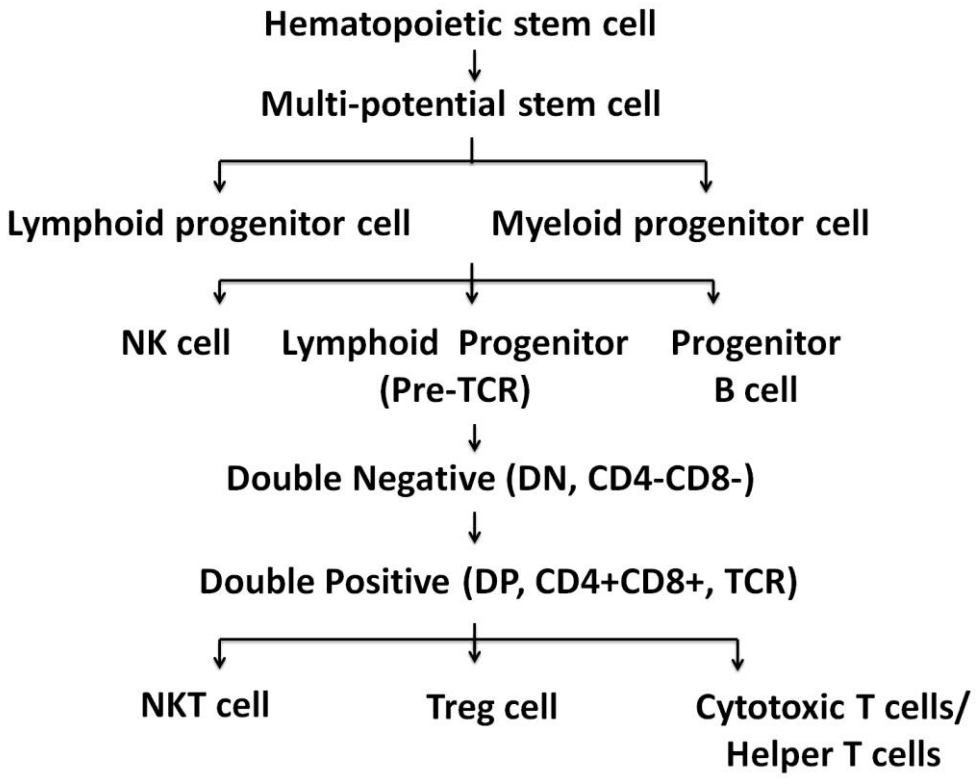



**Figure 1.4 Origins of NK and NKT cells.** Hematopoietic stem cells give rise to all blood lineages. NK cells are innate immune effector cells. They are derivate from either lymphoid or myeloid progenitor cells, and CD3-CD56+. NKT cells are a distinct lineage of T lymphocytes that are derivate from lymphoid progenitor, and CD3+CD56+.

T cells, B cells and NK cells all develop from haematopoietic progenitor cells that reside in the $CD34^{+}$ cell compartment (Lotzova et al., 1993). Figure 1.4 shows the Origins of NK and NKT-like cells in hematopoiesis. Recent studies indicate that bone marrow and lymph nodes are two major physiological sites for NK cell development. IL-2 has been detected as an

effective promoter to support the differentiation of NK cells from $CD34^{+}CD38^{-}$ hematopoietic progenitors (Lotzova et al., 1993, Shibuya et al., 1995). Exogenous IL-2 can also expand NK cell proportions (Caligiuri et al., 1993, Piguet et al., 1986, Rosenberg et al., 1987). IL-15 is another necessary promoter for NK cell development. It has structural similarities with IL-2 and they both share several functions, such as inducing T cell proliferation, generation and activation of NK cells. Studies focusing on the NK cell progenitor in bone marrow have shown that IL-15 plays a critical roles in both the development and the maturation of NK cells (Fehniger and Caligiuri, 2001, Williams et al., 1999). It can induce human $CD34^{+}$ haematopoietic progenitor cell differentiation into NK cells in the absence of other cytokines (Mrozek et al., 1996). However, differentiation based on IL-15 is not able to enhance NK cell expansion (Mrozek et al., 1996), unless two additional bone marrow stromal factors, namely, ligands of the receptor tyrosine kinases c-kit and flt-3, act synergistically with IL-15 (Lyman and Jacobsen, 1998, Yu et al., 1998). Besides bone marrow, lymph node is another important site for NK cell development. $CD56^{bright}$ NK cells have been found in peripheral blood when $CD34^{+}$ progenitor cells are treated with recombinant IL-15 (Lyman and Jacobsen, 1998). Although $CD56^{bright}$ NK cells only comprise around 10% of circulating NK

cells, they are a majority of NK cells (~90%) in lymph nodes. Interestingly, in lymph node, addition of IL-2 can functionally differentiate $CD56^{bright}$ NK cells to $CD56^{dim}$ NK cells through increasing their cytotoxicity (Ferlazzo et al., 2004). Collectively, lymph nodes may serve as a harbour for NK precursor cells that more frequently develop into $CD56^{bright}$ NK cells.

*NK cell receptors*

NK cell receptors can be classified into three families including receptors recognizing MHC class I ligands, receptors interacting with MHC class I related ligands, and receptors for host-encoded non-MHC ligands. NK receptors for MHC class I refer to those with capacities to recognize either classical or non-classical class I molecules encoded by genes within the MHC. In human two representative receptors in this family have been investigated, namely, human killer cell immunoglobulin-like receptors (KIRs) and conserved CD94/NKG2 receptors. These receptors are suggested to play predominant functions in monitoring cells for aberrant expression of MHC class I molecules in immune surveillance.

KIRs, belonging to the Ig superfamily, are also referred to as the CD158 family. They are type I transmembrane glycoproteins that contain two (D1 and D2) or three (D0, D1

and D2) Ig-like domains (designated KIR2D and KIR3D respectively) in the extracellular region (Colonna and Samaridis, 1995, Wagtmann et al., 1995), a short stalk region separating the Ig-like domains from the transmembrane segment, and cytoplasmic domains which characteristics are variable in length, for instance, some receptors possess long (L) cytoplasmic domains containing at least one intracellular immunotyrosine-based inhibitory motif (ITIM) sequence, they are named as KIR2DL or KIR3DL. While other receptors with short (S) cytoplasmic domains possess no ITIM sequence, which are called KIR2DS or KIR3DS. There are also five different types of cytoplasmic tails in the structure of KIRs. Numbers (1-5) designating to which cytoplasmic tail group each KIR belongs are also used to label KIRs, such as KIR2DL1, KIR2DS2 and so on. In humans, 14 different KIR genes have been detected, they are KIR2DL (1-5), KIR2DS (1-5), KIR3DL (1-3) and KIR3DS1 (Raulet et al., 2001). Unlike the ITIM-induced signalling pathway in KIR2DL and KIR3DL, KIR2DS and KIR3DS lacking ITIMs possess a Lys residue in their transmembrane domains. This charged residue associates with an immunoreceptor tyrosine-based activation motif (ITAM)-bearing DAP12 adapter protein to transmit signals. In other words, KIRs with long cytoplasmic domains perform as inhibitory receptors, whereas KIRs possessing

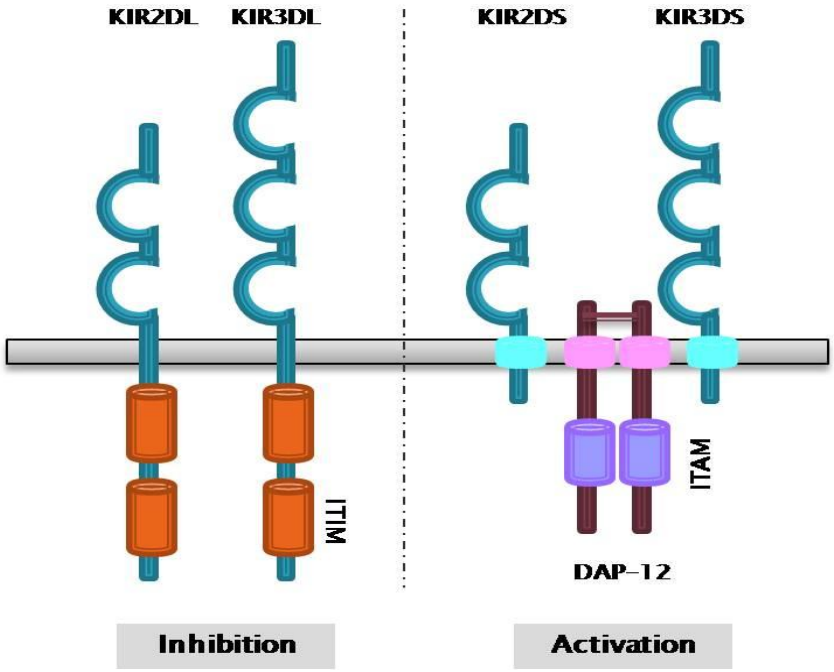



**Figure 1.5 Representative structures of KIRs (KIR2DL, KIR3DL, KIR2DS and KIR3DS).** KIRs contain long (L) cytoplasmic domains possessing at least one ITIM sequence. Thus their major function is inhibitory. Whereas KIRs with short (S) cytoplasmic domains lack ITIMs, they possess a Lys residue in their transmembrane domains. This charged residue associates with an ITAM-bearing DAP12 adapter protein to transmit signals. KIR(S) function as activating receptors. (Adapted from Jordan et al. Natural killer cells in human disease. 2006. Clinical Immunology. 118: 1-10)

short cytoplasmic domains are assumed to be activating receptors. Figure 1.5 shows representative structures of KIRs.

In humans, ligands for KIRs are mainly HLA subfamily, such as HLA-A, HLA-B and HLA-C. KIR2DL can recognize HLA-C

proteins (Colonna et al., 1992), whereas KIR3DL shows the capacity of ligation with HLA-B and certain HLA-A (Gumperz et al., 1997, Gumperz et al., 1995). To date, ligands for KIR2DS and KIR3DS are not known; all studies show that these activation KIRs either do not interact with HLA class I molecules or bind them with much weaker affinity than that of the paired inhibitory KIRs (Valiante et al., 1997, Winter et al., 1998).

KIR2DL4 is a unique gene in KIR family with remarkable structural features. HLA-G is suggested to be KIR2DL ligand in some studies (Boyson et al., 2002, Cantoni et al., 1998, Rajagopalan and Long, 1999).

Most human KIR genes are expressed by subsets of NK cells, memory and effector $CD8^+$ T cells, some $CD4^+$ T cells and $\gamma\delta$ $TCR^+$ T cells except KIR2DL4 (Ferrini et al., 1994, Phillips et al., 1995). Once they are expressed, they are stably maintained on the cell surface. However, KIR2DL4 again as an exception – it is expressed constitutively on the surface of $CD56^{bright}$ NK cells and on only activated $CD56^{dim}$ NK cells (Goodridge et al., 2003).

Interaction of inhibitory KIRs with their MHC class I ligands expressed on target cells can lead to suppression of cytokine production and cytotoxicity of KIR-bearing NK cells and T cells. Although several studies have speculated that lacking an inhibitory receptor for self MHC class I molecule can elicit autoimmune disorders (Namekawa et al., 2000, Snyder et al., 2003, Suzuki et al., 2004, Yen et al., 2001), which is detrimental, KIRs can also be beneficial in immune responses to tumours or viral pathogens (Flores-Villanueva et al., 2001).

CD94/NKG2 receptors are type II transmembrane proteins of the C-type lectin family. They contain two C-lectin domains in the extracellular region, a transmembrane region and a cytoplasmic domain (Lazetic et al., 1996). In humans a single CD94 gene is genetically linked to four NKG2 family genes (NKG2A-, -C, -E, and -F) (Glienke et al., 1998, Houchins et al., 1991). CD94 does not bind ligands of CD94/NKG2 complex and also plays no function on signal transduction (Lazetic et al., 1996, Lohwasser et al., 1999). Unlike KIRs, in humans, CD94/NKG2 receptors recognize non-conventional MHC class I molecules (HLA-E) (Braud et al., 1998).

Although NKG2A and NKG2C share the same ligand HLA-E, their immunological functions are different. Vales-Gomez *et al*

has found that inhibitory receptor CD94/NKG2A shows stronger affinity than the activating receptor CD94/NKG2C. In addition, peptides bound to HLA-E can also affect binding selection between the two receptors (Llano et al., 1998, Vales-Gomez et al., 1999). To date, ligands and functions of CD94/NKG2E and CD94/NKG2F have not been well characterised.

Nearly same as KIRs, CD94/NKG2 receptors are also expressed on most NK cells, memory and effector $CD8^+$ T cells and $\gamma\delta$ $TCR^+$ T cells. However, their expression on $CD4^+$ T cells is undetectable (Lazetic et al., 1996). Also, CD94/NKG2 expression is not constitutively stable and is regulated by surrounding cytokines, such as IL-12, IL-15 and TGF-$\beta$ (Bertone et al., 1999, Derre et al., 2002, Mingari et al., 1998).

Besides KIRs and CD94/NKG2 receptors, leukocyte immunoglobulin (Ig)-like receptors (LILRs) are another type of receptors for MHC class I molecules. One representative member of LILRs family is LILRB1 that has the inhibitory activity. LILRB1 are expressed on a subset of T cells, B cells and monocytes. They can also be found on NK cells from peripheral blood with variable frequency (undetectable to 75%) (Colonna et al., 1997, Cosman et al., 1997). However, its

affinity of ligation with all HLA molecules seems very low (Chapman et al., 1999, Lepin et al., 2000). U18 has been investigated as a ligand with higher affinity to LILRB1 and can elicit their inhibitory activity during viral infection (Beck and Barrell, 1988, Cosman et al., 1997, Chapman et al., 1999).

There is a kind of glycoprotein which is not encoded by genes in the MHC complex but structurally related to MHC class I molecule. It does not serve as a peptide-binding molecule to present antigens to T cells. However, this MHC class I-related protein can be recognized by NK receptor NKG2D (Raulet, 2003). NKG2D, as a type II transmembrane-anchored glycoprotein, is a unique activating receptor which both structurally and functionally differ from other NKG2 receptors (Bauer et al., 1999, Jamieson et al., 2002). It is encoded by a single gene without polymorphism and does not need to form dimers with CD94 (Wu et al., 1999). In human, NKG2D is expressed on the surface of NK cells, $\gamma/\delta$ $TCR^+$ T cells, and especially, it is constitutively present on all $CD8^+$ T cells (Wu et al., 1999). The expression of NKG2D can be regulated by cytokines. For example, IL-15 and TNF-$\alpha$ can enhance the NKG2D expression whereas TGF-$\beta$ causes downregulated effects on it (Castriconi et al., 2003, Lee et al., 2004). Unlike two isoforms of NKG2D (NKG2D-L and NKG2D-S, "L" and "S"

represent long or short protein respectively) found in mouse, humans only express NKG2D-L that transmit signals through transmembrane segment associating with adaptor protein DAP10 (Diefenbach et al., 2002, Wu et al., 1999). Studies demonstrate that in the absence of DAP10, NKG2D is not able to be presented on cell surface and only retained in the cytoplasm (Wu et al., 1999). Human ligands for NKG2D have been identified, such as MICA, MICB, ULBP1, ULBP2, ULBP3 and ULBP4 (Bauer et al., 1999, Cosman et al., 2001). Figure 1.6 presents the interactions of the NKG2D and CD94 family with target cells.

Besides receptors for recognition of MHC class I and MHC class I-like molecules, NK cells also possess additional receptors that can encounter targets lacking expression of MHC class I or MHC class I-related proteins. However, this type of receptors is supposed to be costimulatory receptors because they are unable to individually initiate an immune response. In human, this kind of receptors mainly includes 2B4 receptor (CD244), DNAM-1 receptor (CD226) and CD16 (Fc$\gamma$RIII).

2B4 (CD244) and its ligand CD48 are both members of the CD2 family of Ig-related proteins. In human, they are expressed by all NK cells, most $\gamma/\delta$ TCR$^+$ T cells, memory and

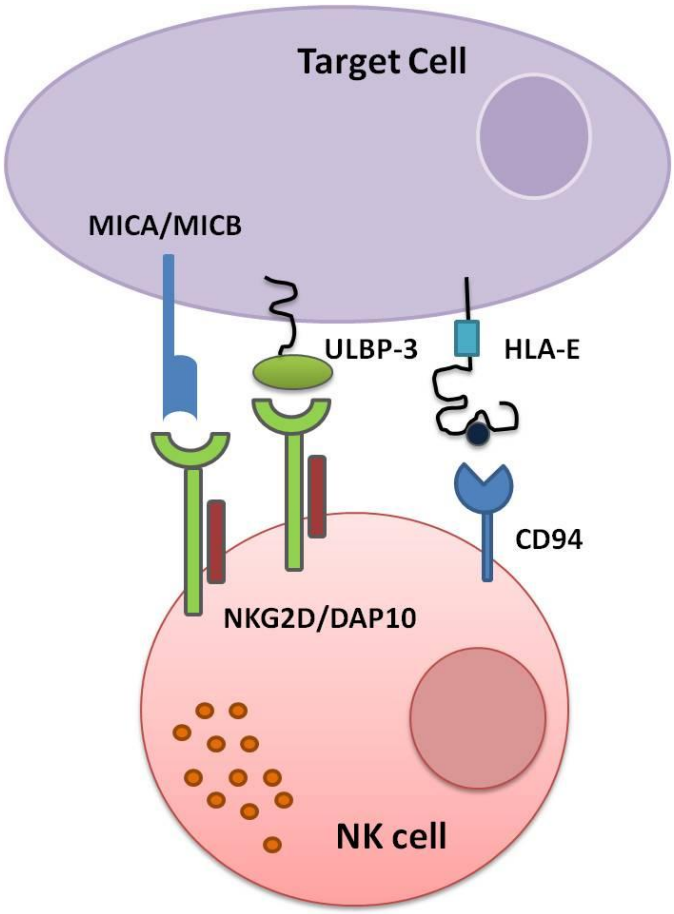



**Figure 1.6 Interactions of NKG2D and CD94 family with target cells.** CD94 receptors are type II transmembrane proteins of the C-type lectin family. In humans a single CD94 gene is genetically liked to four NKG2 family gens (NKG2A-,-C,-E and -F). CD94 receptors recognize non-conventinal MHC class I molecules (HLA-E). NKG2D is a type II transmembrane-anchored glycoprotein. Human only expresses NKG2D-L that transmits signals through transmembrane segment associating with adaptor protein DAP10. Human ligands for NKG2D are MICA, MICB, ULBP1-4.

effector $CD8^+$ T cells, monocytes and basophils (Nakajima et al., 1999, Speiser et al., 2001, Tangye et al., 2000). Although signalling pathways of 2B4 receptor is still unresolved, studies

in human suggest that 2B4 may serve as a multifunctional receptor that can cause either inhibitory or activating responses depending on the stage of NK cell differentiation and activation (Namekawa et al., 2000, Parolini et al., 2000).

DNAX accessory molecule-1 (DNAM-1, also called CD226) is a member of the Ig superfamily and ligated with CD112 and CD155 that are broadly distributed on many tissues (Tahara-Hanaoka et al., 2004). Expression of DNAM-1 is detectable in human NK cells, a majority of T cells, a subset of B cells, monocytes and platelets (Castriconi et al., 2004, Pende et al., 2006, Reymond et al., 2004, Scott et al., 1989, Shibuya et al., 1996).

CD16 is a low affinity receptor for IgG. It binds the lower hinge region of IgG and induces activation signals through FceRI $\gamma$-chain and CD3z, which enable NK cells to mediate antibody-dependent cellular cytotoxicity (ADCC) (Dall'Ozzo et al., 2004, Radaev et al., 2001). Expressions of ICAM-1 together with IgG on infected cells are required to induce efficient ADCC (Bryceson et al., 2005). CD16 shows high expression on $CD56^{dim}$ cells.

In conclusion, since NK cells are defined as non-MHC-restricted cells according to their capacities of recognizing target cells that either express or lack MHC class I molecules, missing-self hypothesis demonstrates that self-MHC molecules engages inhibitory receptors on the surface of NK cells, which can prevent attack against normal cells (Ljunggren and Karre, 1990). Actually, rather than absolutely saying that interaction between MHC-I molecules and inhibitory receptors are terminated, responses of NK cells to target cells are regulated by a balance of signals from inhibitory and activating receptors (Figure 1.7). Either lack of the ligands for inhibitory receptors or signals from activating receptors that are stronger than inhibitory signals can both induce NK cell stimulation. On the other hand, NK cells display their inhibitory functions if there are no activating ligands presented in target cells or much stronger inhibitory signals are avaiable. Several experimental lines of evidence have confirmed this corollary. When activating receptors are sufficiently stimulated or multiple activating receptors are engaged, NK cells still can eliminate infected cells, although MHC class I molecules on these infected cells are still ligated with inhibitory receptors (Cerwenka et al., 2001, Diefenbach et al., 2001, Lanier et al., 1997). Also, Fc-receptors (FcRs) have similar scenarios in immune response. By vitue of coexpression of activating and

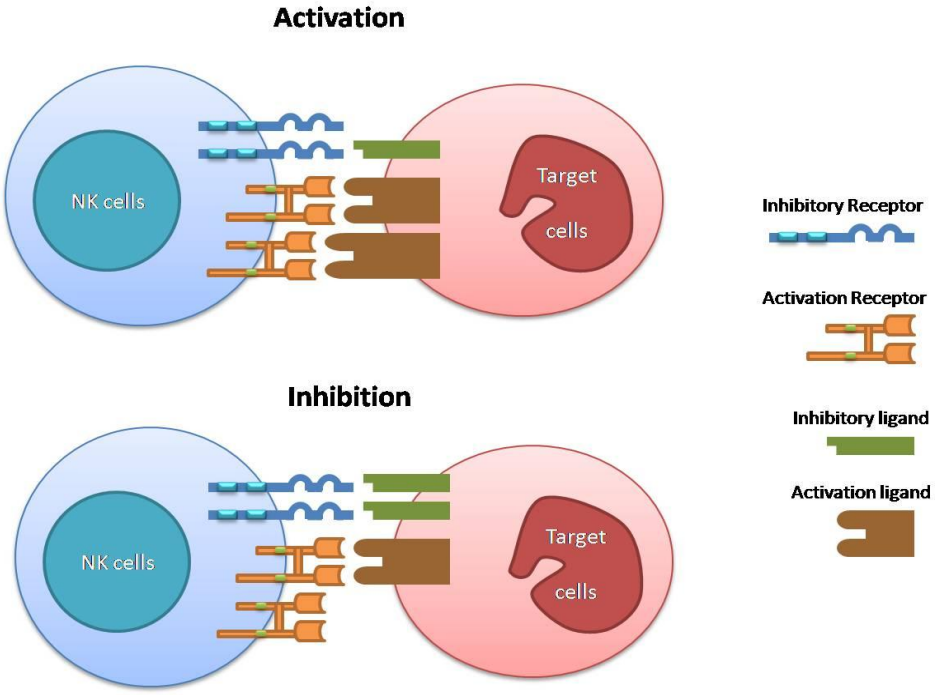



**Figure 1.7 Regulation of NK cell responses by inhibitory or activating receptors.** NK cell activation can be induced in two manners. Either lack of the ligands for inhibitory receptors or signals from activating receptors that are stronger than inhibitory signals can both induce NK cell stimulation. On the other hand, NK cells display their inhibitory functions if there are no activating ligands presented in target cells or much stronger inhibitory signals are available. (Adapted from Lewis L. Lanier. NK cell recognition. 2005. Annu. Rev. Immunol. 23: 225-274)

inihibitory signalling FcRs on the same cells, they set a threshold for immune cell activation by immune complexes. Loss of the balanced signalling leads to uncontrolled response that can result in the damage of healty tissues and ultimately to the initiation of autoimmune processes (Abes et al., 2009).

So far, several major types of NK cell receptors presented in human cells have been introduced with respect to their ligands specificity and signalling properties. However, other receptors expressed on NK cells, such as natural cytotoxicity receptors and cytokine receptors, are also important for generating various NK cell functions in response to target ligands. Although many features and functions of NK cells have been well studied in tumour, viral infection and autoimmune diseases, their immunological properties in COPD patients are still unexplored. Since the predominant risk factor of COPD is cigarette smoking, several studies have successfully detected the effects of cigarette smoke in humans. *In vivo*, previous studies have shown that NK cell numbers and their phagocytic activities are suppressed in both asymptomatic smokers and COPD patients (Ferson et al., 1979, Prieto et al., 2001). Our group further demonstrated that decreased numbers of NK cells are present in peripheral blood from COPD patients compared to healthy non-smokers and healthy smokers

(Urbanowicz et al., 2009). Also, their cytotoxic function is defective. Nevertheless, NK cell proportions and their cytotoxicity are increased in induced sputum from COPD patients in contrast to healthy non-smokers and healthy smokers (Urbanowicz et al., 2010). In addition, cigarette smoke treated NK cells from non-smoking healthy subjects show defective cytotoxicity and their capacity for cytokine secretion is also impaired upon poly I:C activation (Mian et al., 2008). In conclusion, investigation of NK cells' properties in COPD may provide new insights for understanding the development of COPD as well as indicate more beneficial therapeutic approaches for the disease.

#### 1.2.1.7 Epithelial cells

The surface of airway epithelium consists of ciliated cells, goblet cells, ciliated cells and basal cells. These cells work together as an efficient barrier against pathogens through intercellular epithelial junctions. They also exert important roles in airway defence mechanisms, such as clearance of the mucus, water transport, antioxidant, antiprotease functions and cell apoptosis (Puchelle et al., 2006).

Cigarette smoking is the key risk factor in the development of COPD. Injury in airway epithelium caused by cigarette

smoking can result in increased proliferation of airway epithelial cells, which further lead to the squamous metaplasia. Likewise, squamous metaplasia can decrease mucociliary clearance and contribute to the increased risk of squamous cell carcinoma in COPD patients (Cosio et al., 1978, Papi et al., 2004).

A series of studies have shown that cigarette smoking can not only cause the increased expression of IL-8 and mucin in epithelial cells but also reduce the function of trans-epithelial electric resistance (TEER), which reflects a breakdown of the epithelial integrity that can affect the defence mechanisms of the airway (Glader et al., 2006a, Rusznak et al., 1999, Richter et al., 2002, Takeyama et al., 2001). Also, up-regulation of early growth response gene 1 (Egr-1) was detectable when epithelial cells were exposed to cigarette smoke. Egr-1 mediates the production of pro-inflammatory cytokines, such as IL-1$\beta$, TNF-$\alpha$, which have shown increased levels in smoking conditions (Reynolds et al., 2006). Furthermore, *in vitro*, epithelial cells from small airways of healthy smokers and COPD patients secrete more TGF-$\beta$ than do those from healthy non-smokers (Takizawa et al., 2001). Increased levels of fibroblast growth factors (FGF) 1 and 2 are also observed in

the bronchial epithelium of COPD patients (Kranenburg et al., 2005).

Pathogens including bacteria and viruses can exacerbate COPD and accelerate deterioration of lung function (Rohde et al., 2003, Seemungal et al., 2001). Previous studies have shown that rhinovirus (RV) is the dominant viral pathogen in exacerbation of COPD. RV infection can cause secretion of a variety of pro-inflammatory chemokines, such as ENA-78, IL-8 and IP-10, as well as cytokines, for example, IL-1, IL-6, IL-11, IL-16, TNF-$\alpha$ and GM-CSF, by epithelial cells (Proud and Chow, 2006). These inflammatory mediators play different functions on recruitment and activation of inflammatory cells including neutrophils, macrophages, T cells and NK cells that can induce pulmonary and systemic inflammatory responses, mucus hypersecretion and airway remodelling, which result in the exacerbation of COPD. Beside viruses, bacteria can also cause exacerbation of COPD by colonizing and infecting the lower respiratory tract (Murphy, 2006). Although *M. catarrhalis* and H. influenza can trigger immune response in airway epithelial cells (Shuto et al., 2001, Slevogt et al., 2006, Watanabe et al., 2004), a study performed by Sethi *et al*. has shown that alteration in bacterial load was not an important mechanism for exacerbation of COPD (Sethi et al., 2007).

Airway epithelial cells are the initial targets of reactive oxygen species (ROS). Pierrou *et al*. have investigated extensive changes in oxidant response gene expression in the airway epithelial cells that are related to cigarette smoking and the presence of COPD (Pierrou et al., 2007). Furthermore, the levels of 4-hydroxy-2-nonenal-modified (4-HNE) proteins were increased in airway epithelial cells of COPD patients (Rahman et al., 2002). 4-HNE is a diffusible and highly reactive lipid peroxidation end-product. The modification of 4-HNE can lead to abnormal regulation of cell proliferation and inhibition, T cells apoptosis and activation of signalling pathways.

Hypersecretion of mucus and reduction of mucociliary clearance are two main mechanisms inducing mucus accumulation (Danahay and Jackson, 2005, Stannard and O'Callaghan, 2006). The epithelial goblet cells are a main source of secretory gel-forming mucins in the airway. The number of goblet cells has shown increased level in COPD patients compared to healthy smokers (Innes et al., 2006). Mucociliary clearance is dependent on the interactions between the ciliated epithelium, the height of the periciliary fluid and mucus (Stannard and O'Callaghan, 2006). It has been shown that there is significant reduction (nearly no presence) of ciliated cells in COPD patients, compared to

healthy non-smokers and healthy smokers (Rusznak et al., 2000).

Apoptosis is a potent mechanism for elimination of damaged or infected cells. There are abundant studies showing that numbers of apoptotic cells, including bronchial and alveolar epithelial cells and endothelial cells, are increased in the parenchyma in COPD patients (Henson et al., 2006, Hodge et al., 2005b, Hodge et al., 2003a, Park et al., 2007). The apoptosis of airway epithelial cells is disease related rather than smoking status. Hodge S et al. have shown that there is no significant difference in apoptosis of airway epithelial cells between ex-smokers with COPD and current smoking COPD patients (Hodge et al., 2005b), which implies that apoptosis exists after smoking cession.

#### 1.2.1.8 Mast cells

Since neutrophils, macrophages and $CD8^+$ T cells are commonly considered the predominant cells in the pathogenesis of COPD (Chung and Adcock, 2008), the role of mast cells in COPD is just beginning to become unravelled.

Mast cells are highly heterogeneous cells and originate from myeloid stem cells (Anderson et al., 1990). Their maturation

and differentiation are influenced by many cytokines and chemokines, such as IL-3, 4, 9 and 10 (Brown et al., 2008). They are normally classified into two subsets, namely, mucosal mast cells ($MC_T$) and connective tissue mast cells ($MC_{TC}$). The $MC_T$ mainly exist in the respiratory tract and gut, and their number can be modulated in different inflammatory conditions, such as allergy and inflammatory bowel disease. The $MC_{TC}$ are predominantly present in connective tissue, such as the skin, and their number is constant (Irani et al., 1986).

Studies related to the mast cell functions in lung diseases are mostly performed in animal models (Galli et al., 2005, Sekizawa et al., 1989, Thomas et al., 1992). However, recently studies have shown the number, the density and mediators released by mast cells are altered in airways of smokers, even in COPD patients. Wen *et al* have explored that the numbers of mast cells were significantly increased in sputum of smokers compared to ex-smokers (Wen et al., 2010). In the lung and skin of smokers, there is also an increase in absolute numbers of mast cells (Kalenderian et al., 1988). Furthermore, elevated histamine and tryptase levels in BAL fluid from smokers have also been demonstrated (Bessa et al., 2008, Kalenderian et al., 1988). Gosman *et al* have investigated the distribution and the numbers of tryptase- and

chymase- positive mast cells in the airways (Gosman et al., 2008). There was no significant difference in the distribution of these cells in the airways between COPD patients and healthy volunteers. In central airways, the concentration of these mast cells was significantly higher in the subepithelial area, whereas in the peripheral airways, the highest concentration of these cells existed in the adventitia. However, numbers of these mast cells differed between COPD patients and controls. The number of chymase- positive mast cells was significantly higher in both subepithelial areas and smooth muscle of peripheral airways in COPD patients than in controls. Whereas the number of tryptase- positive mast cells in the subepithelial area of central airways was lower in COPD patents than in controls. Moreover, higher percentages of both tryptase- and chymase- positive mast cells were detected in the peripheral airways and positively correlated with $FEV_1$% predicted, which was compatible with the issue that peripheral airways are the main site of airflow limitation in COPD (Hogg et al., 2004, Hogg et al., 1968).

Besides population and distribution, the density of mast cells in the lung of COPD patients has been investigated lately (Hogg et al., 1968). In the small airways, there were significant decreases in total mast cell density in both patients

with severe COPD and patients with moderate to severe COPD compared to healthy non-smokers and healthy smokers. In the pulmonary vessel walls, the reduction of mast cell density was only shown in severe COPD subjects but not other groups. In addition, the balance of MCTC and MCT was shifted. The population of MCT was deceased in all anatomical compartments of the lung, whereas the density of MCTC was markedly higher in the walls of small airways and in alveolar parenchyma in severe COPD groups than other groups. Recently, Soltani and colleague have shown that in COPD airways, both reticular basement membrane and lamina propria mast cells were increased (Soltani et al., 2012)

Taken together, although alternation of mast cell numbers, density and distribution has been identified in several studies, the mechanisms about the signalling pathway and by which mast cells can be activated in the airways of COPD patients are still not well explored. Mast cells express many receptors, such as abundant TLRs, and can release leukotrienes, cytokines and chemokines after activation (Kumar and Sharma, 2010). Therefore, evaluation of expression of key mast cell associated molecules can contribute to more understanding of the role of mast cells in COPD.

#### 1.2.1.9 Eosinophils

Eosinophils are end-stage cells derived from the bone marrow. Their maturation and proliferation are regulated by GM-CSF, IL-3 and IL-5 (Denburg, 1999). Eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO) are two eosinophil specific proteins. They are toxic to bronchial epithelial cells (Blanchard and Rothenberg, 2009). Eosinophils, together with mast cells and basophils, are the predominant source of cysteinyl-leukotrienes (Bozza et al., 1997). Eosinophilic inflammation is mostly studied in allergic airway inflammation (Walsh et al., 2010). Recently, studies on its role in COPD have started to be investigated. Some groups have demonstrated increased numbers of inactive eosinophils in the airways and lavage of stable COPD patients, whereas others have failed to present differences in eosinophil numbers in airway biopsies, BAL, or induced sputum (Lacoste et al., 1993, Turato et al., 2001). Interestingly, the levels of ECP is higher in induced sputum from moderate to severe COPD in the absence of eosinophils (Brightling et al., 2005, Brightling et al., 2000, Gibson et al., 1998, Takahashi et al., 2006), which indicates that eosinophils may have degranulated during disease development (Keatings and Barnes, 1997). A negative correlation between $FEV_1$ and the ratio of activated eosinophils to total eosinophils in endobronchial biopsies has been

reported in COPD patients (Lams et al., 2000). Furthermore, levels of sputum eosinophils and ECP concentration are also negatively correlated with $FEV_1$ (Balzano et al., 1999). Eosinophil function has been accepted to be important in severe COPD. Greater numbers of eosinophils have been detected in bronchial biopsies and BAL fluid during acute exacerbation of COPD compared with stable COPD and controls (Saetta et al., 1996, Saetta et al., 1994).

### 1.2.2 Cytokines in COPD

The potential roles of cytokines in COPD have been explored in many studies. Over 50 cytokines have now been identified in COPD (Barnes, 2004d). However, cytokines work in a complex manner. They may have different or even opposing actions in different cell types or in different inflammatory process.

#### *Proinflammatory cytokines in COPD*

##### 1.2.2.1 Tumour necrosis factor-α (TNF-α)

TNF-α is a member of peptide ligands that stimulate a corresponding series of structurally related receptors (Bazzoni and Beutler, 1996). It plays important roles in many inflammatory diseases. Proinflammatory cells, such as macrophages, monocytes, dendritic cells, B cells, $CD4^+$ T cells, neutrophils, mast cells and eosinophils, and structural cells

including fibroblasts, epithelial cells and smooth muscle cells, are all good sourses for TNF-α production (Russo and Polosa, 2005). Increased levels of TNF-α have been found in induced sputum from COPD patients, pariticularly during exacerbations (Aaron et al., 2001). Soluble TNF-α receptors are also increased in sputum of COPD patients (Vernooy et al., 2002). Serum concentrations of TNF-α are elevated in COPD patients with weight loss, particularly during exacerbations (Calikoglu et al., 2004), which indicates that TNF-α may have functions in the cachexia of severe COPD (de Godoy et al., 1996, Di Francia et al., 1994, Pitsiou et al., 2002). There is a significant increase in TNF-α released from circulating cells in COPD patients compared to normal controls. Plasma concentrations of TNF-α are also increased slightly in COPD patients compared to normal subjects during exercise (Rabinovich et al., 2003).

TNF-α activates NF-κB pathway and p38 mitogen-activated protein kinase (MAPK) that switch on the transcription of inflammatory genes including cytokines, chemokines and proteases released by epithelial cells and macrophages, which indicates that TNF-α play a role in amplifying the inflammation of COPD, such as activation of neutrophils, monocytes, macrophages, epithelium, mucus secretion and destruction of

lung parenchyma (Aggarwal, 2003). Furthermore, TNF-α accounts for 70% of cigarette smoke-induced emphysema in mice (Churg et al., 2004).

#### 1.2.2.2 Interleukin-1β (IL-1β)

IL-1β is a proinflammatory cytokine with similar functions as TNF-α (Watkins et al., 1999). It markedly stimulates alveolar macrophages to produce inflammatory cytokines, chemokines and MMPs in COPD patients (Culpitt et al., 2003). The concentration of IL-1β is increased in induced sputum of COPD patients and is correlated with disease severity. However, concentrations of IL-1 receptor antagonist and soluble IL-1 receptor are reduced in same COPD groups (Sapey et al., 2009).

#### 1.2.2.3 IL-6

IL-6 is a marker of inflammation and plays functions on both proinflammatory response and anti-inflammatory response. Its effects are determined by the presence of other cytokines (Barnes et al., 2011). Therefore, it provides a link between innate and acquired immunity. Increased concentrations of IL-6 are detected in induced sputum, exhaled breath and plasma of COPD patients, especially during exacerbation (Bucchioni et al., 2003, Grubek-Jaworska et al., 2012, Hageman et al.,

2003, Wedzicha et al., 2000). Levels of IL-6 released by monocytes are higher in COPD patients compared to normal subjects (Aldonyte et al., 2003).

#### 1.2.2.4 Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)

GM-CSF is a monomeric protein and is secreted by T cells and macrophages. It is a strong chemoattractant for neutrophils. It also has the ability of enhancing oxidative metabolism and phagocytotic activity of neutrophils and macrophages (Hamilton, 2002). There is a higher concentration of GM-CSF in BAL fluid from stable COPD patients, particularly during exacerbation (Balbi et al., 1997).

#### 1.2.2.5 IL-32

Recently, IL-32, as a newly explored proinflammatory cytokine, has been shown to be induced by IFN-$\gamma$. Upon activation of NF-$\kappa$B and p38 MAPK, IL-32 stimulates the production of TNF-$\alpha$ and IL-1$\beta$ (Joosten et al., 2013). Increased expressioin of IL-32 has been found in epithelial cells, macrophages and $CD8^+$ T cells in COPD patients, and is correlated with disease severity (Calabrese et al., 2008).

#### 1.2.2.6 Thymic stromal lymphopoietin (TSLP)

TSLP is a cytokine belonging to IL-7 family. It regulates the secretion of chemokine ligand by dendritic cells including CCL-17, CCL-22 and CXCL-10, which attract T lymphocytes to the airways (Liu et al., 2007). Increased expression of TSLP has been detected in airway epithelium of COPD patients (Ying et al., 2008). In addition, elevated levels of TSLP can be found in airway smooth muscle of COPD patients (Zhang et al., 2007).

***T cell cytokines***

#### 1.2.2.7 IL-12 family

IL-12, secreted by macrophages, DCs and airway epithelial cells, plays a potent role in Th1 cell differentiation and activation (Watford et al., 2003). It also up-regulates the IFN-$\gamma$ production (Trinchieri and Scott, 1995). Its effects are mostly mediated by activation of STAT4. IL-23, shared p40 subunit with IL-12, plays an important role in regulation of Th17 cells (Qu et al., 2013). It also activates STAT4. IL-27 is another member of the IL-12 family (Bosmann and Ward, 2013, Gee et al., 2009). It has effects on Th1 cell differentiation via a STAT1-dependent mechanism. Increased levels of IL-12 have been detected in bronchial biopsies of COPD patients (Di Stefano et al., 2004). There is also elevated

expression of IL-23 in the bronchial mucosa of COPD subjects (Di Stefano et al., 2009b). A study performed by Huang et al has mentioned that polymorphisms of the IL-27 gene are linked to increased risk of COPD (Huang et al., 2008).

#### 1.2.2.8 Th17 cytokines

Th17 cells are a subset of $CD4^+$ T cells. Their differentiation is regulated by IL-1β, IL-6, and IL-23 (Manel et al., 2008). IL-17A, IL-21 and IL-22 are major cytokines secreted by Th17 cells (Chen and O'Shea, 2008). Traves *et al* have shown increased levels of IL-17A in the sputum of COPD patients (Traves and Donnelly, 2008). Furthermore, the expression of IL-17A, IL-22 and IL-23 are all markedly elevated in the bronchial mucosa of stable COPD patients (Di Stefano et al., 2009b). Interestingly, IL-17A levels are also increased in smokers with normal lung function, which indicates that the secretion of IL-17A is mediated by smoking *per se* rather than disease itself. Increased expression of IL-22 is also observed in the bronchial epithelium and submucosa of COPD patients compared to healthy smokers and healthy non-smokers.

#### 1.2.2.9 Other T cell cytokines

IL-2, IL-4, IL-9, IL-13, IL-15, IL-18 and IFN-γ are all produced by either Tc cells or Th cells (Mosmann and Coffman, 1989).

Higher IL-2 levels have been found in lung plasma samples from stable COPD patients compared to participants with rapidly progressive COPD (D'Armiento et al., 2009). Also, corticosteroid (dexamethasone) has shown less inhibiton of IL-2 production in COPD patients compared to healthy volunteers although these numerical differences between groups did not reach statistical significance (Kaur et al., 2012).

Levels of both IL-4 released by $CD8^+$ T cells and IFN-$\gamma$ released by $CD4^+$ T cells are increased in the BAL fluid from COPD patients (Barczyk et al., 2006). There is also an increase in the expression of IFN-$\gamma$ in bronchial biopsies of COPD patients (Di Stefano et al., 2004a).

IL-9 is normally released by Th2 cells and has functions in allergic inflammation (Shimbara et al., 2000). It increases the expression of MUC5AC and therefore, is a potent inducer of mucus production (Reader et al., 2003). Interestingly, there is a significant increase in the expression of IL-9 in T lymphocytes from bronchial biopsies of COPD patients (Panzner et al., 2003).

IL-13 is another inducer of mucus production and plays a role in the differentiation of goblet cells through activation of EGFR

(Shim et al., 2001). Although there is no significant difference in the expression of IL-13 between COPD and controls (Grubek-Jaworska et al., 2012), smokers with mucus hypersecretion show a higher concentration of IL-13 in bronchial biopsies compared to non-smokers (Miotto et al., 2003).

IL-15 shares several similar functions with IL-2. Details of their features will be indicated in the later chapter. Recently, *in vitro*, it has been found that IL-15 stimulation by itself had no effect on IFN-$\gamma$ protein expression in COPD patients at GOLD stages 2, 3, and 4. However, IL-15 primed $CD8^+$ lung T cells to have greater cytotoxic potential by expressing higher levels of intracellular perforin when they encounter a target cells in COPD (Freeman et al., 2010). Furthermore, CSE treated NK cells were less activated after poly I:C stimulation, and this suppression was IL-15 mediated (Mian et al., 2009).

IL-18 is not only secreted by T cells but also activated macrophages (Nakanishi et al., 2001). IL-18 protein is highly expressed by alveolar macrophages, $CD8^+$ T cells and both the bronchiolar and alveolar epithelia in the lung of COPD patients. Its serum levels are also elevated in smokers with or without COPD compared to non-smokers (Imaoka et al., 2008). IL-18

concentrations are also increased in the induced sputum of COPD patients and correlated with disease severity (Rovina et al., 2009). Higher IL-18 gene expression has been found in plasma and in skeletal muscle of COPD patients (Petersen et al., 2007), which indicates that IL-18 may function in systemic manifestations of COPD.

#### 1.2.2.10 IL-10

IL-10 is an anti-inflammatory cytokine secreted by monocytes, alveolar macrophages and Treg cells. Decreased concentration of IL-10 has been detected in induced sputum of COPD patients (Barnes, 2001). However, its production seems to be elevated in macrophages from normal smokers (Lim et al., 2000). Interestingly, increased expression of IL-10 is found in bronchial biopsies from COPD patients (Panzner et al., 2003).

To sum up, although many cytokines are abnormally expressed in COPD patients, overlapping effects of them mean therapeutic benefits may be trivial. Neverthelss, exploring the cytokine network in COPD provides a new insight for understanding the pathogenesis of disease development.

### 1.2.3 Chemokines in COPD

COPD involves chronic inflammation of small airways and lung parenchyma, with the recruitment of inflammatory cells. Trafficking inflammatory cells to infected areas is orchestrated by multiple chemokines and cytokines. There are over 50 different chemokines that have been indentified. They can selectively activate 19 different surface receptors expressed by target inflammatory cells (Locati et al., 2005). Unlike classical cytokine receptors, receptors for chemokines belong to the 7 transmembrane receptor super family of G-protein-coupled receptors (GPCRs), which makes it possible to discover small molecule inhibitors (Proudfoot, 2002). Four different families of chemokines are now categorized, namely CC, CXC, Cm and CX3C chemokines, based on their differences in the position of critical cysteine residues. Binding of chemokines to their corresponding receptors results in the activation of signal transduction pathways, which further induces chemotaxis or other cellular activities (Gutierrez-Ramos et al., 2000). Currently, several chemokines and their receptors seem to be involved in the pathogenesis of COPD (de Boer, 2005).

#### 1.2.3.1 Interleukin-8

Interleukin-8 (IL-8, CXCL8) is a CXC chemokine and a predominant chemoattractant of neutrophils (Hammond et al.,

1995) . It activates two chemokine receptors, namely, CXCR1 and CXCR2. CXCR1 is a low affinity receptor that is almost specific for IL-8, whereas CXCR2 is a high affinity receptor and is shared by other related CXC chemokines (Premack and Schall, 1996). Binding of IL-8 to these two receptors can activate protein kinase B and GTPases, which consequencely enhances the neutrophil adherence to endothelial cells and directs cell migration. Activated protein kinase B can also stimulate the phosphoinositide 3 kinase that induces F-actin polymerization, leading to pseudopod formation and chemotaxis (Chodniewicz and Zhelev, 2003). In addition, Ras and MAP kinases in neutrophils are activated when IL-8 binds to its receptors, which induces their degranulation (Bowman et al., 1998).

IL-8 is synthesized in several cell types, predominantly macrophages, epithelial cells and neutrophils (Mukaida, 2003), up on activation by several stimuli including TNF-$\alpha$, IL-1$\beta$, bacterial products including LPS, certain viruses, oxidative stress and cigarette smoke extract (DeForge et al., 1993, Johnston et al., 1998, Kwon et al., 1994, Nakamura et al., 1991, Schulz et al., 2004). Release of IL-8 is regulated transcriptionally by several transcription factors, such as NF-$\kappa$B, p38MAP kinase and ERK pathways (Waugh and Wilson,

2008). NF-κB is the predominant transcription factor for IL-8 secretion (Carter et al., 1998). Therefore, inhibition of the NF-κB activating kinase IKK2 results in the reduction of IL-8 production (Hacker and Karin, 2006, Tang et al., 2001).

Previous studies have confirmed that IL-8 is a major chemokine that plays key roles in the neutrophilic inflammation in COPD. The concentration of IL-8 is markedly increased and correlated with increased numbers of neutrophils in both induced sputum and BAL fluid from COPD patients (Keatings et al., 1996, Nocker et al., 1996, Soler et al., 1999, Yamamoto et al., 1997). Unsuprisingly, neutrophils themselves release IL-8 that attracts more neutrophils. Therefore, a self-perpetuating inflammation may be established in COPD patients (Bazzoni et al., 1991). In induced sputum, IL-8 levels are elevated during acute exacerbation and are positively correlated with the bacterial colony count, which indicates that bacterial infection may at least in part induce neutrophilc inflammation through induction of IL-8 secretion in the airways (Hill et al., 2000, Patel et al., 2002). In BAL, IL-8 is the only chemokine with higher concentration in smokers with emphysema, compared to healthy smokers (Tanino et al., 2002). Furthermore, IL-8 concentrations are also increased in hospitalized COPD

patients and are correlated with skeletal muscle weakness (Spruit et al., 2003).

More IL-8 production has been observed in cultured airway epithelial cells and alveolar macrophages from COPD patients than smokers with normal lung function, which also indicates an amplified response (Culpitt et al., 2003, Schulz et al., 2004). Alveolar macrophages provide a great source of IL-8 in response to stimuli. Cells derived from COPD patients release more IL-8 than those from health smokers (Culpitt et al., 2003). IL-8 protein and mRNA are increased in bronchiolar epithelial cells from COPD patients (de Boer et al., 2000). Also, there is elevated basal release of IL-8 from airway epithelial cells of COPD patients (Profita et al., 2003, Schulz et al., 2003).

In clinical trials, anti-IL-8 antibodies decreased only approximately 30% of the neutrophil chemotactic activity of sputum from COPD patients (Beeh et al., 2003, Crooks et al., 2000). This is because other neutrophil chemotactic factors, such as $LTB_4$ and C5a, are also involved. Therefore, blocking IL-8 alone is not sufficient as a therapeutic strategy to reduce neutrophil inflammation in the respiratory tract. CXCR2 is a receptor of IL-8 and is also shared by other chemokines.

Small antagonists of CXCR2 are now in clinical development for the treatment of COPD (Chapman et al., 2009).

#### 1.2.3.2 CXCL1 (growth-related oncogene-α, GRO-α)

CXCL1 is another CXC chemokine that is involved in COPD. It is released by alveolar macrophages and airway epithelial cells upon TNF-α and IL-17 activation (Jones and Chan, 2002, Prause et al., 2003, Schulz et al., 2004). It also binds to CXCR2 expressed by neutrophils and monocytes (Premack and Schall, 1996). CXCL1 concentration is significantly increased in both induced sputum and BAL fluid from COPD patients compared to healthy smokers and healthy non-smokers (Traves et al., 2002). There are greater amounts of CXCL1 released by epithelial cells from COPD patients compared to healthy smokers (Schulz et al., 2004), and by BAL cells from normal smokers compared to non-smokers (Morrison et al., 1998). In COPD patients, monocyte chemotactic activity is increased in response to CXCL1, which may account for the higher amount of alveolar macrophages in the lungs of COPD patients (Traves et al., 2004).

#### 1.2.3.3 CXCL5 (epithelial-cell-derived neutrophil activating peptide-78, ENA-78)

CXCL5 is mainly released by epithelial cells and also binds to CXCR2 (Imaizumi et al., 1997). Unlike IL-8 and CXCL1, CXCL5 does not induce an increased chemotactic activity on monocytes (Traves et al., 2004). The concentration of CXCL5 is increased in the BAL fluid from COPD patients compared to healthy non-smokers (Tanino et al., 2002). However, there is no significant difference between patients with emphysema and healthy smokers. Similarly to CXCL1, the amount of CXCL5 derived from BAL cells is higher in COPD patients compared to healthy non-smokers (Morrison et al., 1998). Furthermore, CXCL5 expression is significantly increased in epithelial cells during exacerbations of COPD (Qiu et al., 2003). Recently, Stefano and colleague showed that the numbers of $CXCL5^{+}$ endothelial cells in the bronchial submucosa is negatively correlated with COPD severity (Di Stefano et al., 2009a).

#### 1.2.3.4 CXCL7 (neutrophil-activating peptide-2)

CXCL7 is released by $\alpha$-granules of platelets but is required to be activated by proteolytic cleavage by enzymes, for example, cathepsin-G (Walz, 1992). It also binds to CXCR2, therefore, it has chemotactic functions for both neutrophils and monocytes. There is an enhanced chemotactic activity of monocytes in response to CXCL7 in COPD patients (Traves et al., 2004). The

number of $CXCL7^+$ cells, including endothelial cells, fibroblasts and submucosal glands in the bronchial submucosa are significantly higher in patients suffering from severe or very severe COPD compared to healthy non-smokers (Di Stefano et al., 2009a).

#### 1.2.3.5 Other CXC related chemokines

Previous studies have shown that T cells, predominantly $CD8^+$ T cells, play a potent role in the pathogenesis of COPD. Homing of T cells to the infected organs depends on initial activation followed by adhesion and selective chemotaxis. CXCL9 (monokine induced by interferon (IFN-$\gamma$), CXCL10 (IFN-$\gamma$-inducible protein of 10kDa, IP-10), and CXCL11 (IFN-inducible T-cell-$\alpha$ chemoattractant) are three chemokines released by IFN-$\gamma$ activated bronchiolar epithelial cells and macropahges. They all can activate CXCR3 expressed by T cells and B cells (Devora, 2000). Studies have shown that CXCL10 expression is increased in bronchiolar epithelial cells and airway smooth muscle cells in COPD patients (Hardaker et al., 2004, Saetta et al., 2002), which leads to the accumulation of $CXCR3^+$ $CD8^+$ T cells (Tc1) and $CD4^+$ T cells (Th1) in the epithelium and submucosa. CXCL11 has the highest affinity for CXCR3 (Clark-Lewis et al., 2003). Its

concentration is also increased in induced sputum of COPD patients.

CXCL12 (stromal-cell-derived factor-1) and CXCL13 (B-cell-attracting chemokine-1) are other two CXC chemokines binding to CXCR4 and CXCR5 respectively (Lapidot et al., 2005). CXCR4 is expressed by T cells, moncytes, immature B cells and airway epithelial cells upon activation. CXCR5 is predominantly expressed by mature B cells, T follicular helper cells (a subset of $CD4^+$ helper T cells) and memory $CD4^+$ T cells (Forster et al., 1996, Kim et al., 2001, Schaerli et al., 2000). Although their roles in COPD have not been explored yet, because their levels are increased in B cells and T cells associated with severe COPD, antagonists of CXCR4 and CXCR5 might be novel treatments for severe COPD.

CXCL16 (scavenger receptor for phosphatidylserine and oxidized low-density lipoprotein) is a transmembrane chemokine expressed by DCs, macrophages and B cells. It binds to CXCR6 expressed by Th1 cells (Kim et al., 2001a). Therefore, it could be relevant to COPD.

#### 1.2.3.6 CCL2 (monocyte chemotactic protein-1, MCP-1)

CCL2 is a CC-chemokine and expressed by alveolar macrophages, T lymphocytes and epithelial cells. It is also released by type II pneumocytes in culture. Its secretion is triggered by LPS but inhibited by CSE. CCL2 activates CCR2 on monocytes and lymphocytes (Rose et al., 2003). Previous studies have shown that CCL2 levels are enhanced in induced sputum, BAL fluid and lungs of COPD patients (Capelli et al., 1999, de Boer et al., 2000, Traves et al., 2002). Recently, CCL2 expression has also been detected in small macrophages in induced sputum from COPD patients. Interestingly, there is a significantly increased level of mRNA expression of CCL2 in COPD patients compared to healthy non-smokers. Similar results are also seen in healthy smokers, although it is less pronounced (Frankenberger et al., 2010). TLR-2 and TLR-4 can down-regulate CCR2 expression (Parker et al., 2004). Blocking CCR2 induces the termination of the chemoattractant effect of induced sputum from COPD patients (Monzon et al., 2011), which indicates that antagonism of CCR2 may be a useful therapeutic strategy in COPD.

#### 1.2.3.7 Other CC-chemokines

CCL3 (Macrophage inflammatory protein, MIP-1$\alpha$) and CCL4 (MIP-1$\beta$) are secreted by macrophages, taken together with CCL5 (released by activated normal T cells expressed and

secreted, RANTES), they all not only activate CCR1 expressed by monocytes and neutrophils, but also bind to CCR5 expressed on Th1 and Tc1 cells (Devora, 2000). The levels of CCL3, CCL4 and CCL5 are all increased in COPD patients (Barnes, 2009). Numbers of $CCR5^+$ T lymphocyte are elevated in induced sputum of COPD patients and in the airways of mild-moderate COPD patients but is decreased in severe disease (Costa et al., 2008, Di Stefano et al., 2001, Grumelli et al., 2004). CCL5 expression is increased in the airways and sputum of COPD patients during exacerbations, which indicates a role of CCR5 in the recruitment of T cells and macropahges during exacerbations (Fujimoto et al., 2005, Zhu et al., 2001a). In addition, CCL5 also activates CCR3 predominantly expressed by eosinophils.

CCL7 (MCP-3) and CCL13 (MCP-4) are CC-related chemokines and bind to CCR2 and CCR3 respectively. CCL17 (thymus- and activation-dependent chemokine, TARC), CCL20 (MIP-3$\alpha$) and CCL22 (macrophage-derived chemokine) are members of CC-chemokines and recruit target cells by interaction with CCR8, CCR6 and CCR4 respectively (Devora, 2000). Similar to CCL2, mRNA expression of CCL7, CCL13 and CCL22 are all markedly increased in small macrophages from induced sputum of COPD patients and healthy smokers compared to healthy non-

smoking subjects, although less pronounced in smokers (Frankenberger et al., 2010). CCL20 is a potent chemoattractant of dendritic cells and is mainly expressed by airway epithelial cells in response to IFN-γ (Reibman et al., 2003). Levels of CCL20 protein are increased in induced sputum from COPD patients compared to never smokers and smokers without COPD, and are negatively correlated with $FEV_1$% function. There is also a significant inverse correlation between CCL20 mRNA expression in total lung and $FEV_1$% (Demedts et al., 2007).

#### 1.2.3.8 $CX_3C$ chemokines

$CX_3CL1$ (fractalkine) is a unique $CX_3C$ chemokine that is expressed on cell surfaces. Its expression is enhanced in human airway epithelial cells after IFN-γ induced stimulation. It is involved in the recruitment and adhesion of monocytes, T lymphocytes and natural killer cells to epithelial cells (Fujimoto et al., 2001). However, its role in COPD is unexplored. Recently it has been implicated in the function of $CX_3CL1$ in dendritic cell recruitment, which might be relevant to antigen presentation in COPD (Niess et al., 2005).

To sum up, cytokines and chemokine play potent roles in the pathogenesis of COPD. Figure 1.8 shows the pathological hallmarks of COPD.

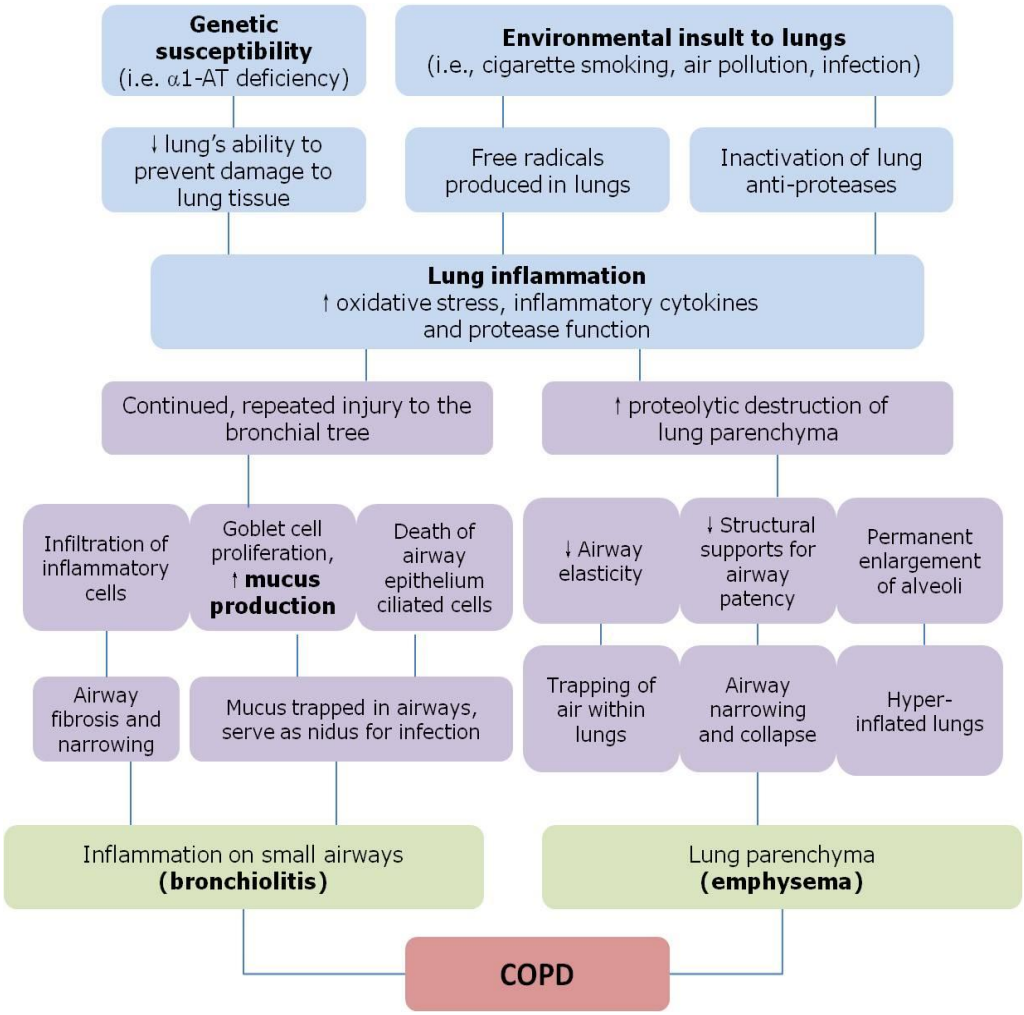



**Figure 1.8** Pathological hall marks of COPD. The three main pathological hallmarks of COPD are chronic obstructive bronchiolitis, emphysema and mucus hypersecretion.

### 1.2.4 Oxidative stress in COPD

Excessively production of ROS can generate oxidative stress, which results in damage to lipids, proteins and DNA (Finkel, 2003). Oxidative stress can also lead to the oxidation of arachidonic acid and the formation of isoprostanes (a new series of prostanoid meidators), which have functional effects including bronchoconstriction and plasma exudation. Activated neutrophils, eosinophils, macrophages and epithelial cells are all good sources for ROS production in the airways (MacNee, 2001a). For examples, in neutrophils, hydrogen peroxide ($H_2O_2$) generated from superoxide anions ($O_2 \bullet^-$) is metabolized to hypocholorous acid in the presence of chloride ions, which is strong endogenous oxidant in the lung (Eiserich et al., 1998). Cigarette smoke, nitrogen dioxide, and ozone are exogenous oxidants that have the capacity to enhance the oxidative stress in the lungs (Pryor and Stone, 1993, Sunyer, 2001). In human airways, the production of oxidants is controlled by several antioxidant mechanisms (Cantin et al., 1990). However, the major antioxidants are extracellular, such as glutathione peroxidase that is significantly up-regulated in response to cigarette smoking and oxidative stress (Avissar et al., 1996).

There are abundant studies showing increased oxidative stress in COPD (MacNee, 2001b, Repine et al., 1997). Increased xanthine/xanthine oxidase activity has been observed in BAL fluid from COPD patients (Pinamonti et al., 1998). 4-hydoxy-2-nonenal is a specific marker for lipid peroxidation that is detectable in lungs of COPD patients and is location specific (Rahman et al., 2002). Ethane formed through lipid peroxidation is elevated in the breath of COPD patients and its concentration is correlated with disease severity (Paredi et al., 2000). Concentrations of $H_2O_2$, 8-isoprostaglandin $F_{2\alpha}$, malondialdehyde and thiobarbituric acid reactive substances are all increased in exhaled breath condensate of COPD patients (Montuschi et al., 2000). Formation of ROS can activate NF-$\kappa$B and activator protein-1 transcription factors, which induce a neutrophilic inflammation through increased expression of IL-8 and other chemokines, TNF-$\alpha$ and MMP-9 (Rusznak et al., 1996). Therefore, this evidence indicates that increased oxidative stress in the respiratory tract plays a potent role in the disease by amplifying the inflammatory and the destructive responses in COPD.

### 1.2.5 Nitric oxide in COPD

Peroxynitrite is an oxidant and nitrating agent. It can damage a wide array of molecules in cells, including DNA and proteins

(Pacher et al., 2007). Increased oxidative stress leads to the formation of peroxynitrite via combination of nitric oxide (NO) with superoxide anions and then nitrate, which both change the gaseous phase of NO. This may explain why the NO levels are reduced in normal smokers (Kharitonov et al., 1995). However, in COPD patients, the NO levels are increased and are positively correlated with eosinophil numbers and bronchodilator response (Papi et al., 2000). The enzyme inducible NO synthase (iNOS) that generates NO is expressed in macrophages and lung parenchyma of patients with COPD, especially in severe COPD (Ichinose et al., 2000, Maestrelli et al., 2003). Furthermore, concentrations of nitrate are also elevated in breath condensate and sputum of smokers with or without COPD (Corradi et al., 2001, Kanazawa et al., 2003). Increased levels of NO are detected in the alveolar compartment of COPD patients and correlates with disease severity (Brindicci et al., 2005). This corresponds to the finding of increased inducible NO synthase in the periphery of lungs in COPD subjects (Maestrelli et al., 2003). Therefore, the peripheral NO may be a useful non-invasive biomarker of COPD inflammation (Barnes et al., 2006).

### 1.2.6 Peptide mediators in COPD

Endothelin-1 (ET-1) and bradykinin are two kinds of peptide related to COPD development (Barnes, 2004d). ET-1 is a potent vasoconstrictor with the ability to cause vascular smooth muscle hyperplasia (Bouallegue et al., 2007). Giaid *et al* have shown that expression of ET-1 is increased in pulmonary endothelial cells of COPD patients who have secondary pulmonary hypertension (Giaid et al., 1993). ET-1 concentrations are also increased in induced sputum and BAL fluid in COPD patients (Bacakoglu et al., 2003, Chalmers et al., 1999). All these findings indicate that ET-1 secretion may play an important role in pulmonary vasoconstriction and pulmonary hypertension in COPD. Bradykinin is a potent bronchoconstrictor of huma airways, particulary small airways (Hulsmann et al., 1994). It can stimulate mucus secretion and potentiates cough in airways (Nagaki et al., 1996). Although its function in COPD is not fully explored, proinflammatory cytokines produced in COPD increase the expression of bradykinin receptors in pulmonary cells (Tsukagoshi et al., 1995).

### 1.2.7 Lipid mediators in COPD

#### 1.2.7.1 Prostaglandin $E_2$ ($PGE_2$) and Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)

$PGE_2$ is a bronchodilator of human airways (Pavord and Tattersfield, 1995). It inhibits the release of proinflammatory cytokines from monocytes and of acetylcholine from airway cholinergic nerves (Meja et al., 1997, Spicuzza et al., 1998). Therefore, $PGE_2$ may have beneficial effects in COPD. However, it can also stimulate mucus secretion and expression of mucin genes (Borchers et al., 1999), and activate airway sensory nerves to enhance coughing (Stone et al., 1992). $PGF_{2\alpha}$ is a bronchoconstrictor and has the ability of activating airway sensory nerves to produce cough (Nichol et al., 1990). Increased concentrations of both $PGE_2$ and $PGF_{2\alpha}$ are detected in exhaled breath of COPD patients (Montuschi et al., 2003).

#### 1.2.7.2 Leukotriene $B_4$ ($LTB_4$)

$LTB_4$ is mainly released from activated alveolar macrophages and neutrophils in the airways (Shamsuddin et al., 1997). It is a potent chemoattractant of neutrophils and binds to its receptors, namely, $BLT_1$ and $BLT_2$, expressed on neutrophils and T lymphocytes respectively (Yokomizo et al., 2000). Increased levels of $LTB_4$ have been demonstrated in both exhaled breath condensate and induced sputum from patients with stable COPD (Beeh et al., 2003, Woolhouse et al., 2002), and particularly enhanced during exacerbations (Biernacki et al., 2003).

### 1.2.8 Growth Factors in COPD

The release of growth factors can induce fibrosis and cell proliferation and further cause structural changes in small airways and lung parenchyma.

#### 1.2.8.1 Transforming growth factor-β (TGF-β)

TGF-β is a multifunctional growth factor with the ability to induce proliferation of fibroblasts and airway smooth muscle cells, deposition of extracellular matrix and epithelial repair. It also has immuno-suppressive effects on Th1, Th2 and Th17 cells (Yoshimura and Muto, 2011). Increased expression of TGF-β has been detected in airway epithelial cells and macrophages from small airways of COPD subjects (Takizawa et al., 2001). TGF-β levels are also elevated in peripheral blood monocytes from COPD patients (Hodge et al., 2003b). In peripheral lung tissue from COPD patients, TGF-β expression is increased and is correlated with immunoreactivity for 4-hydroxy-4-nonenal that is a marker of oxidative stress (Rahman et al., 2002).

#### 1.2.8.2 Epidermal growth factor (EGF)

EGF, together with TNF-α, activates EGF receptor (EGFR) (Argast et al., 2004, Lee et al., 2007a). It plays a critical role

in regulation of mucus secretion, differentiation of mucus-secreting cells and expression of mucin genes (Burgel and Nadel, 2004). Increased levels of EGF, EGFR and the related cytokine, heregulin, have been detected in airway epithelial cells of COPD patients (Ganesan et al., 2012).

#### 1.2.8.3 Vascular endothelial growth factor (VEGF)

VEGF is a regulator for the growth of new vessels and vascular leakage (Ho and Kuo, 2007). Its expression is increased in pulmonary vascular smooth muscle of patients with mild and moderate COPD but is reduced in severe COPD with emphysema (Santos et al., 2003). The level of VEGF is also reduced in induced sputum and the lung of COPD patients (Kanazawa and Yoshikawa, 2005).

#### 1.2.8.4 Fibroblast growth factors (FGF)

FGF is related to vascular remodelling. Increased expression of FGF-1 and FGF-1 receptor, and FGF-2 have been investigated in large pulmonary vessels and small pulmonary vessels respectively (Kranenburg et al., 2005).

### 1.2.9 Proteases in COPD

#### 1.2.9.1 Matrix metalloproteases (MMPs)

MMPs are a large family of up to 26 homologous endopeptidases, which can not only degrade protein components of the extracellular matrix but also has a potent function as regulating secretion of cytokines, chemokines and growth factors (Verma and Hansch, 2007). They are secreted by stromal cells and two major inflammatory cells including neutrophils and alveolar macropahges (Betsuyaku et al., 1999, Finlay et al., 1997a). Imbalance between proteinase and antiproteinase can lead to extracellular matrix destruction in the airways and contributing to airway remodelling and to decreased lung function in patients (Abboud and Vimalanathan, 2008). Therefore, the balance between MMPs and tissue inhibitors of MMPs (TIMPs) is thought to be crucial in disease pathogenesis (Barnes, 2002).

There are abundant studies showing the role of MMPs in COPD patients. MMP-9 is released by bronchial epithelial cells, neutrophils, alveolar macrophages and eosinophils (Atkinson and Senior, 2003). Its expression is induced by IL-13 and inhibited by TNF-$\alpha$ and IL-1$\beta$. There are feedback loops between MMP-9 and TGF-$\beta$, and bewteen MMP-9 and IL-8 (Fini et al., 1998). The expression of MMP-9 on alveolar macrophages is elevated in the BAL fluid from patients with emphysema (Finlay et al., 1997b). Increased levels of MMP-9

and its inhibitor TIMP-1 are also detected in induced sputum from stable COPD patients, which correlates with both neutrophilia and $FEV_1$, compared to controls (Cataldo et al., 2000, Culpitt et al., 2005, Vernooy et al., 2004). In COPD exacerbations, levels of MMP-9 are markedly increased in induced sputum and are positively correlated with numbers of neutrophils and lymphocytes, but not macrophages (Mercer et al., 2005). Moreover, higher expression of MMP-9 is found in alveolar macrophages from healthy smokers than healthy non-smokers (Russell et al., 2002a).

A variety of experimental models of COPD have been established to detect effects of MMP-12 in COPD. Emphysema induced by cigarette exposure is prevented in MMP-12 knockout mice (Hautamaki et al., 1997). Also, MMP-12 -/- mice do not develop to the lavage neutrophilia as seen after smoke exposure. However, the neutrophil influx can be restored by instillation of wild type alveolar macrophages (Churg et al., 2003). Unlike findings in mice, studies on MMP-12 expression in human are controversial. Some studies demonstrate that MMP-12 mRNA is hardly detected in the lung (Gosselink et al., 2010, Imai et al., 2001, Lee et al., 2010), whereas others do show its difference between COPD patients and controls. For example, MMP-12 levels are higher in

induced sputum from stable COPD patients, no matter whether they are current smokers or ex-smokers, compared to healthy smokers and healthy non-smokers (Demedts et al., 2006, Ilumets et al., 2007). Its enzymatic activity is also significantly higher in COPD subjects. These data indicate that MMP-12 in sputum is not induced by cigarette smoking per se but by the disease itself. Babusyte *et al* show that the number of MMP-12 positive macrophages is higher in BAL fluid from current smokers with COPD than ex-smokers with COPD and controls (Babusyte et al., 2007). Another group also found the MMP-12 levels are higher in the macrophages from current smokers compared to ex-smokers (Wallace et al., 2008).

MMP-1 is also called interstitial collagenase-1. It is produced by bronchial epithelial cells, type II pneumocytes and alveolar macrophages in the lung (Verma and Hansch, 2007). Increased levels of MMP-1 mRNA and protein are detected in the lung of COPD patients (Gosselink et al., 2010, Lappalainen et al., 2005, Selman et al., 2003).

In addition, levels of MMP-2 are elevated in induced sputum, lung parenchyma and lung tissue of COPD patients (Gueders et al., 2006, Ramos et al., 2009, Segura-Valdez et al., 2000). Also, increased levels of MMP-8 are detected in induced

sputum, particlularly during exacerbations in COPD patients (Ilumets et al., 2007, Skillrud et al., 1986, Vernooy et al., 2004).

Taken together, MMPs account for most of the elastolytic activity released from alveolar macrophages and neutrophils in COPD patients. They not only contribute to the destruction of extracellular matrix but also play important roles in small airway remodelling and emphysema.

#### 1.2.9.2 Neutrophil elastase (NE)

NE is a serine protease that is inhibited by anti-protease $\alpha$1-antitrypsin in the lung parenchyma. It is stored in neutrophils and expressed on the cell surface when cells are activated by cytokines (Owen et al., 1997). NE is a potent mucus secretagogue of submucosal gland cells and goblet cells (Sommerhoff et al., 1990). It induces the expression of MUC5AC (Fischer and Voynow, 2002, Voynow et al., 1999). It also induces secretion of cytokines and chemokines, such as IL-8 (Nakamura et al., 1992). The apoptosis induced by macrophages are also impaired by NE (Vandivier et al., 2002). The amount of NE/$\alpha$1-AT complexes is increased in BAL fluid

from current smokers with COPD and is negatively correlated with $FEV_1$ (Betsuyaku et al., 2000, Yoshioka et al., 1995).

## 1.3 Role of apoptosis in the pathogenesis of COPD

Unlike necrosis that is an uncontrolled process, apoptosis, programmed cell death, plays a key function in the elimination of unwanted damaged or infected cells (Baetu and Hiscott, 2002). Three major pathways have been involved in the regulation of apoptosis, namely, ligand-death receptor pathway (e.g, TNF-$\alpha$ and Fas ligand), cytolytic effector cell pathway (e.g, perforin and granzymes) and the mitochondrial intrinsic pathway (e.g. bcl-2). All pathways finally converge at the level of caspase-3 activation. Caspase activation leads to DNA fragmentation, characteristic of apoptotic cells and the cleavage of cytoskeletal proteins (Plataki et al., 2006). Figure 1.9 shows the different mechanisms of apoptosis induced by cytotoxic cells.

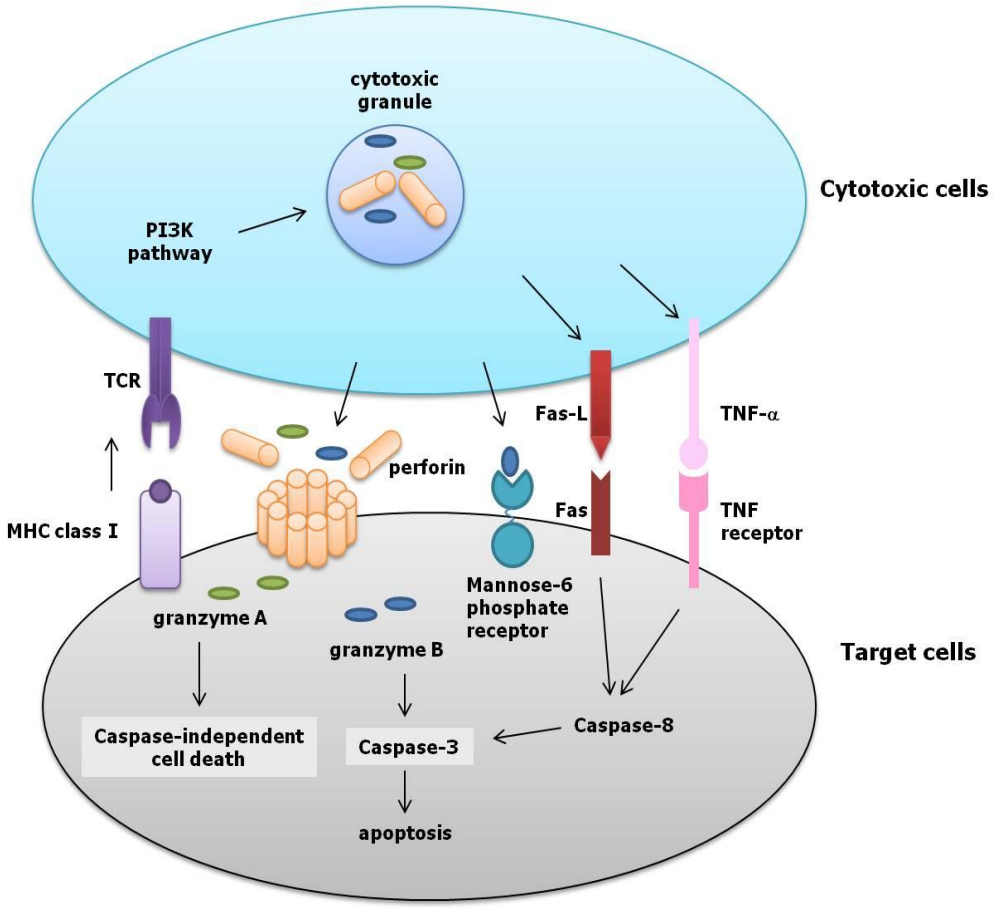



**Figure 1.9 Different mechanisms of apoptosis induced by cytotoxic cells.** Infected cells (Target cells) present antigen peptides to cytotoxic cells by binding to their TCRs. Activated cytotoxic cells then release granule killing molecules, such as perforin and granzymes. The former one is traditionally considered to form pores on the cytoplasm of target cells which facilitates granzymes passage. However, others indicate the granzyme B can still be endocytosised independently of perforin by mannose-6 phosphate receptors. Besides granule molecules, Fas-L and TNF-α expressed by cytotoxic cells interact with Fas and TNF receptor expressed by target cells respecitively. Grznzyme A and granzyme B induce cell apoptosis in different manners.

Studies on apoptosis in COPD are well established. Segura-Valdez et al were the first to show increased apoptosis in lung tissue sections of COPD patients compared to controls (Segura-Valdez et al., 2000). Both apoptosis and proliferation of cells (alveolar epithelial cells, endothelial cells and mesenchymal cells) have been elevated in the lung tissue of patients with emphysema compared to healthy subjects with or without smoking history (Imai et al., 2005, Yokohori et al., 2004). VEGF is a growth factor that induces the expression of anti-apoptotic proteins and is a survival factor of endothelial cells (Alon et al., 1995, Gerber et al., 1998). Its levels are decreased in induced sputum of COPD patients and correlated with disease severity. Moreover, both the percentage and absolute number of apoptotic bronchial epithelial cells were significantly higher in COPD patients, with increased expression of Fas, p53 and activated caspases (Hodge et al., 2005a). Reduced apoptosis of neutrophils was found in hospitalized patients with an acute exacerbation of COPD (Pletz et al., 2004).

In peripheral blood studies, several mediators involved in the induction of T cell apoptosis, such as TNF-$\alpha$ and Fas, are upregulated in COPD patients (Hodge et al., 2003b). There is a marked increase in soluble Fas in plasma from severe COPD

patients but not in mild or moderate COPD and healthy controls (Yasuda et al., 1998). Since COPD is a complex lung disease with systemic consequences, skeletal muscle apoptosis has been studied. Interestingly, the skeletal muscle apoptosis is elevated in COPD patients who have a low BMI (Agusti et al., 2002).

The major mechanism of cytotoxic cell-mediated killing is performed by cytotoxic granules and Fas-FasL (Russell and Ley, 2002). After recognition of target cells by effector cells, granules released by cytotoxic cells move to the immunological synapse by exocytosis. Then their membrane fuses with the killer cell plasma membrane and release contents into target cells by endocytosis (Hayes et al., 1989, Stinchcombe et al., 2001). A pore forming protein perforin and a group of serine proteases named as granzymes are two representative types of granules related to granule-mediated cytolysis (Pasternack et al., 1986, Podack et al., 1985).

### 1.3.1 Perforin

Perforin is homologous to the complement protein C9. It is expressed by all cytolytic cells including cytotoxic T cells, NK cells and NKT cells. Traditionally, perforin is thought to form pores in the target cell plasma membrane in a $Ca^{2+}$-dependent

manner that help granzymes to pass through (Tschopp et al., 1986). Indeed, pores are not big enough to allow passage of globular molecules as big as granzymes. Sublytic concentrations are required to deliver granzymes (Kawasaki et al., 2000, Metkar et al., 2002). Increased expression of perforin and granzyme B has been detected on $CD8^+$ T cells from BAL fluid in COPD patients and healthy smokers compared non-smokers (Hodge et al., 2006). Percentages of killer cells expressing both perforin and granzyme B were also markedly higher in induced sputum of COPD groups (Chrysofakis et al., 2004, Urbanowicz et al., 2010). Morissette *et al* have shown that there is no significant difference in the percentage of $CD8^+$ or $CD4^+$ T lymphocytes and NK cells expressing perforin from peripheral blood between emphysematous smoking COPD patients and control groups (Morissette et al., 2007). Whereas Urbanowicz *et al* have demonstrated proportions of killer cells expressing both perforin and granzyme B are lower in the peripheral blood from COPD patients (Urbanowicz et al., 2009).

### 1.3.2 Granzymes

Granzymes are a family of highly homologous serine proteases. Their main function is to induce cell death to eliminate infected cells. Also, they play a role in immune regulation

(Regner and Mullbacher, 2004). Five human granzymes have been identified, namely, A, B, H, K, M (Barry and Bleackley, 2002). However, the most abundant granzymes are granzyme A and B, which induce cell death through alternate and nonoverlapping pathways.

*Granzyme A*

Granzyme A is the most abundant protease in the cytotoxic granules (Garcia-Sanz et al., 1990, Gershenfeld and Weissman, 1986). It is mainly expressed by NK cells and activated cytotoxic T lymphocytes. Unlike perforin and other granzymes, granzyme A is continuously expressed after T cell activation, perhaps even in long-term memory cells (Chen et al., 2001, Zhang et al., 2003). Although it is also delivered into target cells via perforin, it initiates innate immune response via a granzyme B and caspase independent pathway and has the extracellular matrix cleavage capacities including degradation of type IV collagen (Chowdhury and Lieberman, 2008, Shresta et al., 1999). Interestingly, a recent study shows that, rather than its cytotoxic ability, the major role of granzyme A is promoting inflammation through inducing production of pro-inflammatory cytokines in monocytic cells (Metkar et al., 2008). Increased expression of granzyme A, but not granzyme B, has been detected in type II

pneumocytes in patients with severe COPD (Vernooy et al., 2007).

*Granzyme B*

Granzyme B is a serine protease and is classically considered to be expressed by cytotoxic T lymphocytes and NK cells (Fairclough et al., 2008). Recently, it has also been found in many different types of myeloid cells that are noncytotoxic, such as Treg cells, type II pneumocytes, basophils, macrophages, DCs, smooth muscle cells and so on, generally without perforin (Gondek et al., 2005, Grossman et al., 2004). Therefore, besides the major function in inducing cell death, granzymes also have additional non-cytolytic functions, for examples, regulating lymphocyte survival and immune tolerance, promoting inflammation and potentially enhancing lymphocyte migration (Chowdhury and Lieberman, 2008). Tranditionally, perforin is thought to be essential in mediating the action of granzyme B against target cells. Perforin forms a multimeric transmembrane pore in the target cell, which facilitates granzyme B passing into the target cells (Bossi and Griffiths, 2005). Recently, another perforin-independent model of this process has been established. Granzyme B is endocytosed in complex with the mannose-6 phosphate receptor while perforin has not been expressed by effector

cells (Froelich et al., 1996, Metkar et al., 2002, Shi et al., 1997). Granzyme B mediated apoptosis is caspase-dependent. Caspase 3 is a central execution caspase and is directly processed by granzyme B (Talanian et al., 1997, Yang et al., 1998). In addition, activation of T cells induces the expression of perforin and granzymes. IL-2 is the only stimulus that consistently upregulates the expression of granzyme A, granzyme B and perforin (Liu et al., 1989). IL-15 and IL-21 also activate granzyme B and perforin expression in $CD8^+$ T lymphocytes from human peripheral blood samples (White et al., 2007).

The specific role of granzyme B in COPD is much more studied than other granzymes. Hodge *et al* have shown increased expression of granzyme B and perforin by $CD8^+$ T cells from BAL fluid in COPD patients and healthy smokers compared to non-smokers (Hodge et al., 2006), and there was a correlation between the percentage of granzyme B expressing T cells and bronchial epithelial cell apoptosis. In the same study, both soluble granzyme B and the percentage of T cells with intracellular granzyme B have been observed to be increased in COPD patients. Percentages of $CD8^+$ T lymphocytes, NK cells and NKT-like cells expressing both perforin and granzyme B were markedly higher in induced sputum of COPD groups

(Urbanowicz et al., 2010). Nevertheless, findings produced using peripheral blood samples are complicated. Morissette *et al* have reported that there is no significant difference in the percentage of $CD8^+$ or $CD4^+$ T lymphocytes and NK cells expressing granzyme B between emphysematous smoking COPD patients and control groups including healthy non-smokers and healthy smokers (Morissette et al., 2007). Conversely, intracellular granzyme B has been investigated in a greater percentage of T cells from the peripheral blood of COPD patients (Hodge et al., 2006). Studies performed in our group have shown that the percentage $CD8^+$ T lymphocytes, $CD6^{dim}CD16^+$ NK cells and NKT-like cells that expressed both perforin and granzyme B were significantly lower in COPD patients compared with the healthy smokers and non-smokers (Urbanowicz et al., 2009).

*Granzyme K*

Granzyme K is another tryptase and causes cell death in a capase-independent manner similar to granzyme A. However, it is expressed much less than granzyme A. Granzyme K is mainly expressed by activated killer cells and a small population of $CD4^+$ T cells (Bratke et al., 2008, Hameed et al., 1988). Although there is no difference in granzyme K expression in BAL fluid from COPD patients and allergic

asthma (without allergen provocation), it is indeed upregulated in cases of acute airway inflammation (Bratke et al., 2008). These findings suggest that granzyme K release is a specific event in acute airway inflammation, and chronic inflammation, such as stable COPD, does not equate to a pan-granzyme effect and that there may be inter-granzyme variability regarding activity and leves in different diseases.

*Other human granzymes*

Up to date, there are no studies on the role of granzyme H and granzyme M in COPD.

### 1.3.3 Fas and Fas-ligand

Fas is a transmembrance receptor belonging to the TNF receptor family. It is widely expressed on normal cells and malignant cells (Leithauser et al., 1993, Nagata and Golstein, 1995, Smith et al., 1994). Fas ligand (Fas-L) is a membrane-bound cytokine belonging to the TNF family. It is mainly expressed on activated T cells and induces cell apopotosis by binding to Fas (Suda et al., 1995, Suda et al., 1993). Unlike levels of TNF-$\alpha$ that are significantly higher in peripheral blood from COPD patients, there is no significant difference in the serum level of soluble Fas-L and the plasma level of soluble Fas among groups (Takabatake et al., 2000). Interestingly,

another study has shown that the proportion of Fas positive T lymphocytes is dramatically higher in peripheral blood from COPD patients compared to healthy non-smokers and smokers. Moveover, the proportion of $CD8^+$ T cells expressing Fas is correlated with disease severity (Domagala-Kulawik et al., 2007). Increased expression of Fas is also detected in bronchial epithelial cells in COPD subjects (Hodge et al., 2005a).

### 1.3.4 Granulysin

Granulysin is a proinflammatory molecule expressed by activated cytotoxic T cells and natural killer cells (Krensky and Clayberger, 2005). It is also a chemoattaractant for monocytes, T lymphocytes, and activates the expression of many cytokines, such as IL-1, IL-6 and IL-10 (Deng et al., 2005). Studies have shown that granulysin has broad clinical relevance to a myriad disease including cancers (Kishi et al., 2002), skin afflictions (Ammar et al., 2008), infections (Sahiratmadja et al., 2007) and reproductive complications (King et al., 2003). However, it is still to be studied in COPD.

## 1.4 Hypothesis of this project

Chronic Obstructive Pulmonary Disease (COPD) is a chronic inflammatory disease involving both innate and adaptive immune responses. Activated inflammatory cells can release inflammatory mediators, such as TNF-$\alpha$, IFN-$\gamma$, matrix-metalloproteinase and IL-8, that sustain the inflammatory process and finally lead to airway remodelling (Chung, 2005), parenchymal destruction and mucocilialary dysfunction (Danahay and Jackson, 2005). Activated neutrophils (Damiano et al., 1986, Galdston et al., 1977), macropahges (Molet et al., 2005, Montano et al., 2004) and DCs (Demedts et al., 2007) have been observed in the airways or sputum of COPD groups. $CD8^+$ T-lymphocytes, NKT-like cells and NK cells are the three main types of killer cells in the immune system and have been implicated in the pathogenesis of COPD (Fairclough et al., 2008). Abnormal numbers of $CD8^+$ T-lymphocytes have been found in large airways sub-epithelium (O'Shaughnessy et al., 1997, Lams et al., 2000), peripheral airway (Saetta et al., 1998), parenchyma (Saetta et al., 1999), smooth muscle (Baraldo et al., 2004), bronchial arteries (Peinado et al., 1999, Saetta et al., 1999, Turato et al., 2002), peripheral blood (Urbanowicz et al., 2009), sputum samples (Urbanowicz et al., 2010), and small airways such as broncheoalveolar lavage (BAL) (Saetta et al., 2002, Saetta et al., 2003). Abnormal proportions of NKT-like cells have also been detected in

peripheral blood (Urbanowicz et al., 2009), and induced sputum (Urbanowicz et al., 2010) from smokers with COPD. The frequency of iNKT cells is lower in peripheral blood from patients with exacerbations of COPD compared to stable COPD patients (Chi et al., 2011). Reduced numbers of NK cells and their phagocytic activities have been observed in peripheral blood from both asymptomatic smokers and COPD patients (Ferson et al., 1979, Prieto et al., 2001). However, NK cell proportion and their cytotoxicity are increased in induced sputum from COPD patients in contrast to healthy non-smokers and healthy smokers (Urbanowicz et al., 2010). Taken together, all these findings indicate that killer cells may be much more activated in COPD groups and play an important role in pathogenesis of disease. Figure 1.10 shows how NK cells interact with other leucocytes and epithelims to become activated.

This project we are aiming to explore immunological features of killer cells in COPD, especially under the condition of cigarette smoking induced activation. Main approaches of this project are as followed. Firstly, we compared the activation status of killer cells *ex vivo* in both healthy participants and

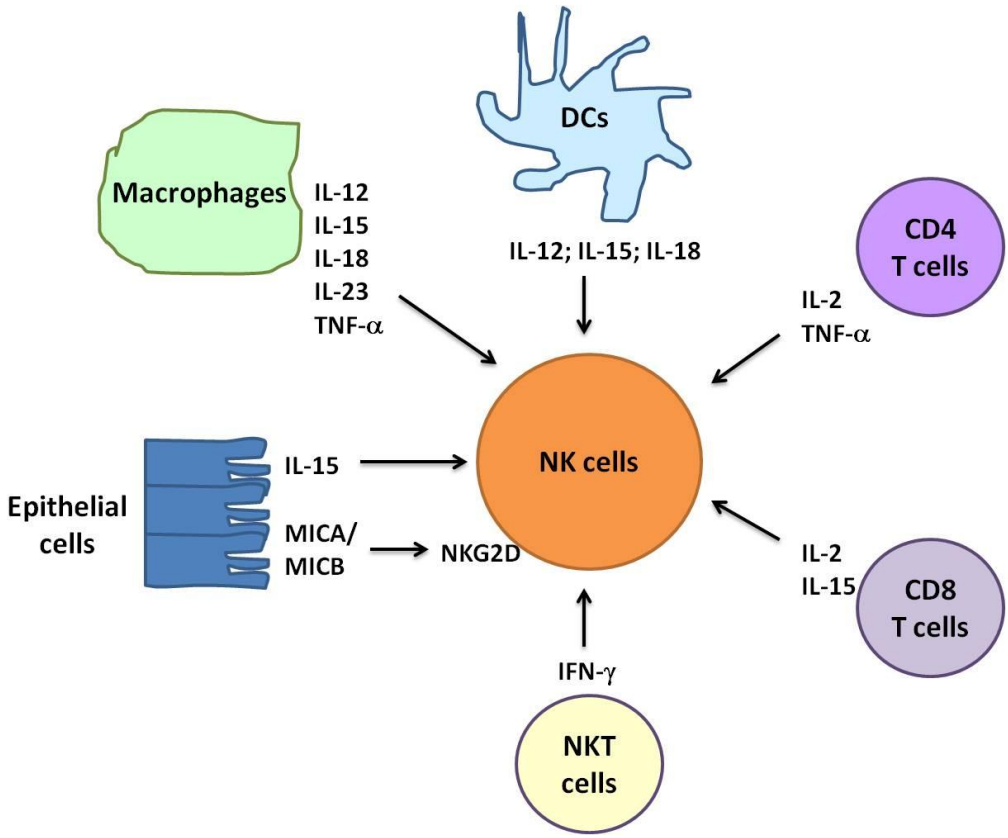



**Figure 1.10 Interaction between NK cells and other lymphocytes.** NK cells are the innate immune cells expressing multiple receptors. Activation of NK cells can be induced either by interaction of ligands to functional receptors, such as MICA to NKG2D; or by cytokines, for example, IL-2, 15, 18, 23, INF-γ and TNF-α, produced by other lymphocytes. Epithelial cell is a big source of producing IL-15.

COPD patients. Cigarette smoking is still the predominant risk factor in COPD, we hypothesis that cigarette smoke extract may induce immunological changes on killer cell functions. Therefore, *in vitro*, effects of cigarette smoke extract on killer cell activation upon different stimulation were investigated between study groups. Expression levels of effoctor molecules, such as granzymes, perforin and Fas-ligand, by killer cells

were also tested. Furthermore, cytotoxicity of $CD8^+$ T cells in each study group was also investigated. All above research were performed in peripheral blood samples. Finally, we compared the activation levels of killer cells in BAL from COPD patients.

# Material and Methods

## 2.1 Study population and procedures

The study population consisted of 16 healthy non-smoking volunteers, 12 healthy smokers, and 21 COPD patients who were either current smokers or ex-smokers. The diagnosis of COPD was made according to the ATS guidelines. Healthy non-smokers and current healthy smokers were with an $FEV_1$>80% of predicted normal and had normal lung function. COPD patients had irreversible airflow limitations on spirometry ($FEV_1$<80% of predicted with $FEV_1$/FVC ratio of <70%) and were reversibility to an inhaled beta-2 agonist of<10% or <200 mls absolute improvement. Smokers suffering from COPD accrued at least a 20-pack year smoking history and healthy smokers had a similar smoking history to them. All participants were age-matched and met the following inclusion criteria. Firstly, they were nonatopic (negative skin prick test response to common allergen extracts including grass pollen, house dust mite, cat dander and dog hair). Secondly, there was also no personal or family history of allergic diseases with them, such as allergic rhinitis. Thirdly, these subjects did not have any history of systemic or other pulmonary diseases, for example, TB, asthma, lung cancer, bronchiectasis or $\alpha$1-anti-trypsin deficient. In addition, they should also not be infected by acute viruses or bacteria. Finally, all COPD patients had been free of an acute exacerbation of COPD for at least 6

weeks preceding the study and none had received antibiotics or corticosteroids (oral and inhaled) over the same period.

Other four COPD patients were recruited for the BAL study. The inclusion/exclusion criteria were the same as mentioned above.

The study protocol was approved by the Nottingham Research Ethics Committee (REC reference 04/Q2403/102) and informed consent was obtained from all participants before entering the blinded study.

## 2.2 Peripheral Blood Mononuclear Cell (PBMCs) isolation

Peripheral venous blood (50mls) from each participant was diluted in warm RPMI 1640 (Sigma, Poole, UK) with 100U/ml penicillin and 100ug/ml streptomycin (RPMI-1640 medium) at 2:1 ratio and then layered over a discontinuous Histopaque density gradient (Sigma, Poole, UK). PBMCs were removed from the surface of Histopaque after centrifugation at 800g for 22 minutes and washed twice in RPMI medium at 300g, 4°C for 5 minutes. Isolated PBMCs were resuspended in appropriate medium. Prior to experiments, cells were counted with a Hemocytometer using standard protocol. Cell viability

was assessed with 0.5% Trypan Blue (Sigma, Poole, UK) staining.

## 2.3 $CD56^+$ and $CD8^+$ cell separation

Isolated PBMCs were incubated with anti-CD56 MicroBeads (Miltenyi Biotech Ltd, Surrey, UK) in MACS buffer containing sterile D-PBS (Sigma), 1% heat-inactivated fetal calf serum (FCS) (QB-Perbio, UK) and 0.4% EDTA (Sigma, Poole, UK) for 15 minutes at 4°C. $CD56^-$ cell fractions were obtained by cell suspension passing through a cold MS column attached to a magnet. After washing three times, the column was removed from the magnet and 1ml MACS buffer was added and quickly plunged through the MS column into sterile tube for collection of $CD56^+$ cells. To obtain NKT-like cells ($CD3^+CD56^+$) and NK cells ($CD3^-CD56^+$), $CD56^+$ cells were incubated with Multisort Release Reagent for 10 minutes at 4°C, washed and then incubated with MultiSort Stop Reagent and $\alpha$-CD3 microbeads for 15 minutes at 4°C. Finally the labelled cells were separated on a refrigerated LS column. $CD8^+$ T lymphocytes were positively selected from CD56 negative fraction after incubated with anti-CD8 MicroBeads (Miltenyi Biotech Ltd, Surrey, UK) by using the same method of NKT-like cell separation. All isolated fractions were washed and counted. Detailed protocol for cell separation was shown on

manufacturer's instructions. The purity of $CD3^+CD56^+$, $CD3^-$ $CD56^+$ cells or $CD8^+$ $CD56^-$ cells was over 90%, as verified by flow cytometry.

## 2.4 Cigarette smoke extract (CSE) preparation

It should be noted that there are more than four thousand components in cigarettes that contribute to the diversity of cigarette brands. Choosing Marlboro to produce cigarette smoke extract in this project is because, of all menthol and non-menthol cigarettes, Marlboro is the brand used most often by 42.4% of smokers (Ref, office of applied studies. The NSDUH report Cigarette Brand Preferences in 2005. 2007).

A CSE collection apparatus is set up prior to 30 minutes of cell culture for each experiment in fume hood. As shown in Figure 2.1, the chemicals and equipment for the apparatus are as followed. (1) 10ml of Phenol-red free RPMI-1640 (GIBCO, Invitrogen, Paisley, UK) in a 30ml steralin; (2) cigarette (Marlboro, EU; Tar 10mg, Nicotine 0.8mg, carbon monoxide 10mg) with filter cut off; (3) 3-way tap; (4) two sections of flexible tubing; (5) steralin lid with hole cut in, for tubing to fit through; (6) 50ml syringe to aspirate the smoke; (7) two clapmps and one clamp stand.

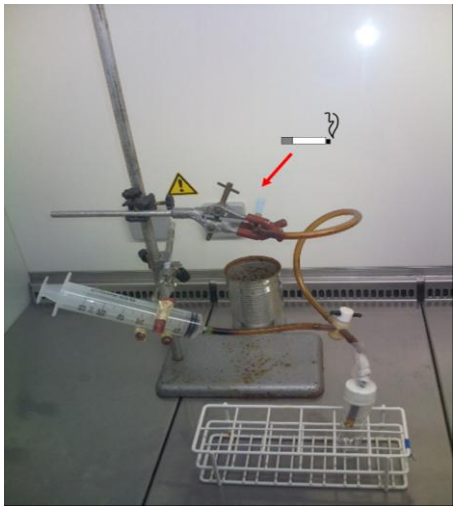


**Figure 2.1 Apparatus of CSE preparation**

To collect CSE, firstly, the tap should be switched to the position that keep the tubing leading to the seralin is off. CSE from single lit commercial cigarette were aspirated to the 50ml syringe at rate of 125ml in 60s. Then, switch the tap so that the route to the steralin containing 10ml of Phenol-red free RPMI-1640 is ON. Slowly bubble the entire 50ml of smoke into the medium. Repeat this process until the cigarette is finished. The crude CSE was filtered through a 0.45um pore filter to remove bacteria and/or large particles and subsequently adjusted with medium to an absorbace reading of 0.15 at 320nm. This solution was taken as 100% CSE.

## 2.5 Cell culture and treatments

Isolated PBMCs, NKT-like cells, NK cells or $CD8^+CD56^-$ T lymphocytes cells were resuspended in RPMI-1640 medium supplemented with 10% FCS at a density of $1x10^6$ cells/ml. Cell suspension was loaded to a 96-well flat bottom tissue culture plate at 200ul of each well. To detect the cell activation, receptor expression and cytokine secretion, cells were stimulated with anti-CD3 (R&D System, Abingdon, UK), recombined human IL-2 (rhIL-2; PeproTech, EC, London, UK), recombined human IL-15 (rhIL-15; PeproTech, EC, London, UK), and CSE and incubated at 37°C in 5% $CO_2$. The time period of incubation and concentrations of these stimulators were determined by each experiment protocol. Specially, anti-CD3 was pre-coated in the plate for 90 minutes at 37°C in 5% $CO_2$ and wash in cold sterile D-PBS twice to remove the unbound antibody.

For cytotoxicity assay, isolated $CD8^+CD56^-$ cells were only incubated for 24 hours at 37°C in 5% $CO_2$. Before cell incubation, the plate was coated with 2μg/ml anti-CD3 (in 50ul D-PBS solution each well) for a minimum of 2 hours at 37°C in 5% $CO_2$. Target cells (P815 cells) were grown in RPMI-1640 medium supplemented with 10% FCS at 37°C in 5% $CO_2$ and ready for use when they reached confluence.

## 2.6 Extracellular staining and Intracellular staining

To harvest maximal cells for analysis, cells in each well were flushed with 200ul PBA (PBS; 1% BSA; and 0.1% sodium azide; Sigma, Poole, UK) twice and washed with 1ml PBA in a FACS tube (Elkay, Hampshire, UK). For extracellular staining, cells were incubated with fluorescently conjugated antibodies at the recommended concentrations for 30 minutes at 4°C. Unlabeled antibodies were removed with 1ml PBA washing up twice. Control tubes with single colour fluorescently conjugated antibody were used for setting compensation on flow cytometer by technician. Additional tubes with fluorescence minus one (FMO) were set up for quadrant setting in data presentation.

To detect the intracellular protein secretion, prior to 10 hours of harvesting, cells were treated with 10ug/ml brefeldin A (Sigma, Poole, UK). After washed with 1ml PBA solution, cell pellets were fixed in 3% formaldehyde in isotonic azide free solution, incubated for 5 minutes at 4ºC, centrifuged, washed with 1ml PBA, 1ml PBA with 0.04% saponin (Sigma, Poole, UK) and 1ml PBA with 0.04% saponin and 10% FCS orderly. Interested labeled antibodies were mixed to cells at recommended concentrations and cells were incubated on a

rocker at 4°C for 2 hours (see table 1). Unbound antibodies were removed by 1ml PBA with 0.04% saponin washing up twice.

All centrifugations were performed at 300g for 5 minutes. Finally, cell pellets were fixed in 0.5% formaldehyde in isotonic azide free solution and stored at 4°C for flow cytometry analysis.

## 2.7 $CD8^+$ T lymphocytes Redirected Cytotoxicity Assay

The cytotoxicity of $CD8^+$ T lymphocyte was measured with colorimetric quantification of lactate dehydrogenase LDH released from lysed target cells (P815 cells). Harvested $CD8^+$ $CD56^-$ T cells were counted in a standard manner and resuspended at $4x10^6$ cells/ml in RPMI-1640 medium without phenol red and FCS. P815 target cells were treated with 10ug/ml of anti-CD3 and incubated at 37°C. After 30 minutes incubation, P815 cells were washed twice and resuspened in RPMI-1640 without phenol red supplement with 10% FCS at a density of $1x10^5$ cells/ml. A U-bottom 96-well culture plate was used in this protocol.

RPMI-1640 without phenol red supplement with 5% FCS was loaded to two triplicate sets of wells at 100ul/well. The top three wells, called as Culture Medium Background, were established to correct for contributions caused by phenol red and LDH activity that may be present in serum containing culture medium. The bottom three wells, called as Volume Correction Control, were used for correcting for the volume increase caused by addition of Lysis Solution (10X).

Target cells were added at 50ul/well to two triplicate sets of wells each containing 50ul RPMI-1640 without phenol red. The top three wells were used for measurement of target cell spontaneous LDH release, the bottom three was for the detection of target cell maximum LDH release.

Four effector to target (*E:T*) ratios were tested in this assay, namely, 40, 20, 10 and 5. Diluted effector cells at 50ul were loaded to each well containing 50ul of RPMI-1640 without phenol red supplement with 10% FCS. Target cells at 50ul were mixed with 50ul of diluted $CD8^+$ T cells. Both conditions were prepared in triplicates as well.

Effector and target cells were brought into close contact by centrifuging the plate at 250g for 4 minutes and incubated for

4 hours at 37°C in 5% $CO_2$. Forty-five minutes prior to harvesting supernatants, wells used for Volume Correction Control and target cell maximum LDH release were treated with Lysis Solution (10X) at 10ul/well. After 4 hours incubation, plate was centrifuged at 250g for 4 minutes. 50ul aliquots from all wells were transferred to a fresh 96-well flat-bottom (enzymatic assay) plate. Assay buffer Substrate Mix (Promega, USA) was thawed at 37°C shortly and added to each well at 50ul to detect LDH activities. The plate was then covered with foil and incubated for 30 minutes at room temperature. After incubation, 50ul of Stop Solution (Promega, USA) was added to each well and the sample was read at 49nm on an Emax precision microplate reader (Molecular Devices, Workingham, UK) using SOFTmax software (Molecular Devices) immediately. The percentage of cell-mediated cytotoxicity was calculated by the following formula:

Cytotoxicity (%) = {(effector/target cell mix-spontaneous effector LDH release-spontaneous target LDH release)/(maximum target LDH release-spontaneous target LDH release)} x 100

## 2.8 BAL preparation

Bronchoscopy with BAL was performed by a clinician from the Queen's Medical Centre, University of Nottingham, UK. Four COPD patients were recruited to this research. Each of them was sedated with oxycodon and scopolamine hydrobromide 10 minutes prior to the procedure. A fibreoptic bronchoscope (Olympus 1T 20 D, Olympus Corporation, NY, USA) was pushed in the subsegmental bronchus of the right middle lobe via the nasal route. Four 50ml aliquots of sterile, pre-warmed normal saline were administered and sucked into four plastic bottles by gentle hand suction through the bronchoscope. All bottles were kept in ice. The first pooled samples were discard. Other three samples were used for BAL cellular analysis.

Before centrifugation of BAL fluid, two 50ml centrifuge tubes were weighted on an electronic scale. Samples were pipetted into the tubes and the volumes of them were noted. Filled tubes were re-weighted and the weight of recovered BAL fluid was calculated. A sterile piece of the 48um nylon gauze was put in a 35mm sterile funnel to pour through BAL samples, and then the volume and the weight of filtrate were measured and noted. Filtrated BAL fluid was centrifuged at 400g for 10 minutes at 4°C. The cell pellets were washed twice with sterile D-PBS by centrifugation. Total cell counts were determined

with a haemocytometer. Cells were resuspended in RPMI medium supplemented with 10% FCS. To determine the cell populations in the BAL, extracellular staining was performed for these cells as described previously.

## 2.9 Flow cytometric analysis

Labelled cells were analyzed on an EPICS ALTRA flow cytometer (Beckman Coulter, Buckinghamshire, UK). For each test, 50000 total events were collected, unless otherwise stated. Data analysis was performed using WEASEL software version 2.3 (The Walter And Eliza Hall Institute of Medical Research, Melbourne, Australia). For each staining experiments, compensation detection was set up by using control tubes with single colour fluorescently conjugated antibody. Fluorescence minus one (FMO) tubes were also used for quadrant setting. Dead cells were excluded according to forward scatter vs side scatter characteristics.

## 2.10 Statistical analysis

The statistical analysis was performed with Prism software, version 4.0c (GraphPad). Data that were non-normally distributed are expressed as median and range, unless otherwise stated. Normality was detected using the Kolmogorov–Smirnov test. Differences between the four

groups of participants were tested using the non-parametric Kruskal-Wallis test with *post hoc* pairwise comparisons made by the Dunn's Multiple Comparison test to determine which pair was statistically significantly different. Differences between the "before and after" treatment were tested by parametric stuent's *t* test. Confidence interval 99% was used to show two-tailed p values. P values of less than 0.05 or 0.01 were considered to indicate statistical significance according to the size of each study group.

## 2.11 Antibodies for flow cytometry

Table 2.1 shows all antibodies used in whole project. FITC, PE and PC7 labelled antibodies were added at 5ul to cell pellets; ECD and PC5 were added at 2.5ul.

**Table 2.1 Antibodies for extracelluar staining and intracellular staining**

| Antigen | Fluorochrome | Isotype | Clone | Source |
|---|---|---|---|---|
| CD3 | ECD<br>PC7 | Mouse IgG1 | UCHT1 | Beckman Coulter, Luton, UK |
| CD4 | FICT<br>PC5 | Mouse IgG1 | 13B8.2 | Beckman Coulter, Luton, UK |
| CD8 | PC5 | Mouse IgG1 | B9.11 | Beckman Coulter, Luton, UK |
| CD8 | ECD | Mouse IgG1 | SFCl21Thy2D3 | Beckman Coulter, Luton, UK |
| CD25 | FITC<br>PE | Mouse IgG2a, k | B1.49.9 | Beckman Coulter, Luton, UK |
| CD56 | PE<br>PC5 | Mouse IgG1 | N901 | Beckman Coulter, Luton, UK |
| CD69 | ECD<br>PC5 | Mouse IgG2b | TP1.55.3 | Beckman Coulter, Luton, UK |
| KIR3DL1 (CD158e1) | FITC | Mouse IgG1, k | DX9 | Biolegend, San Diego, CA, USA |
| NKG2D (CD314) | PE | Mouse IgG1 | ON72 | Beckman Coulter, Luton, UK |
| GranzymeB | FITC | Mouse IgG1, k | GB11 | BD Pharmingen™ |
| Perforin | PE | Mouse IgG2b, k | δG9 | BD Pharmingen™ |
| Fas-L (CD178) | PE | IgG Hamster | 4A5 | Beckman Coulter, Luton, UK |

# Results

## 3.1 Activation of killer cells in peripheral blood in COPD patients *ex vivo*

### 3.1.1 Introduction

Many risk factors have been implicated in COPD, but cigarette smoking is still considered the common and key pathological driver in the development and progression of COPD (Mannino and Buist, 2007). Smoking cessation has shown great potential in control of disease development (Scanlon et al., 2000, Tashkin and Murray, 2009).

$CD8^+$ T-lymphocytes, NKT-like cells and NK cells are the three main types of killer cells in the immune system and have been implicated in the pathogenesis of COPD (Fairclough et al., 2008). Abnormal numbers of $CD8^+$ T-lymphocytes have been found in large airways sub-epithelium (O'Shaughnessy et al., 1997, Lams et al., 2000), peripheral airway (Saetta et al., 1998), parenchyma (Saetta et al., 1999), smooth muscle (Baraldo et al., 2004), bronchial arteries (Peinado et al., 1999, Saetta et al., 1999, Turato et al., 2002), peripheral blood (Urbanowicz et al., 2009), sputum samples (Urbanowicz et al., 2010), and small airways such as broncheoalveolar lavage (BAL) (Saetta et al., 2002, Saetta et al., 2003). Abnormal proportions of NKT-like cells have also been detected in

peripheral blood (Urbanowicz et al., 2009), and induced sputum (Urbanowicz et al., 2010) from smokers with COPD. The frequency of iNKT cells is lower in peripheral blood from patients with exacerbations of COPD compared to stable COPD patients (Chi et al., 2011). Reduced numbers of NK cells and their phagocytic activities have been observed in peripheral blood from both asymptomatic smokers and COPD patients (Ferson et al., 1979, Prieto et al., 2001). However, NK cell proportion and their cytotoxicity are increased in induced sputum from COPD patients in contrast to healthy non-smokers and healthy smokers (Urbanowicz et al., 2010). In addition, cigarette smoke treated NK cells from non-smoking healthy subjects have shown defective cytokine secretion upon poly I:C activation (Mian et al., 2008).

Studies on $CD4^+$ T cells are less extensive than $CD8^+$ T cell infiltrates. $CD4^+$ T cells have been shown to be increased in airways and lung parenchyma in COPD patients (O'Shaughnessy et al., 1997, Saetta et al., 1999), particularly in proximity to bronchus associated lymphoid tissue (Hogg et al., 2004). $CD4^+$ helper T cells are mainly responsible for orchestrating downstream immune process by producing cytokines. They are essential for the full development of

adaptive immune cytotoxicity by priming and promoting the long-term survival of $CD8^+$ T cells.

Activation is a prerequisite for killer cells to be functional. Activated killer cells can cause the production of inflammatory cytokines, chemokines and cytotoxic molecules, such as INF-$\gamma$, TNF-$\alpha$, IL-8 and granzymes, which can induce pathological features including emphysema, mucus hypersecretion and cell apoptosis, and lung tissue damage in COPD (Barnes, 2004d). Several studies have investigated expression of activation markers on T-lymphocytes from peripheral blood, induced sputum and bronchial alveolar lavage (BAL) fluid from healthy smokers and COPD patients with varying results (Glader et al., 2006b, Leckie et al., 2003), but none have looked specifically at $CD8^+$ T lymphocytes, NKT-like and NK cells. CD69 and CD25 are cell surface markers of early and late stages of lymphocyte activation, respectively (Caruso et al., 1997). Killer cell activation involves several factors, such as interaction between antigen receptors and peptides presented by MHC class I molecules or MHC class I-related proteins. Human killer cells express immunoglobulin-like receptors (KIRs), belonging to the Ig superfamily (CD158 family), which bind to MHC Class I. They can be classified into two groups, namely, inhibitory KIRs and activating KIRs. Inhibitory KIRs

possess long (L) cytoplasmic domains, such as KIR2DL or KIR3DL. Activating KIRs possess short cytoplasmic domains with an immunoreceptor tyrosine-based activation motif (ITAM)-bearing DAP12 adapter protein (Parham, 2005). NKG2D is a unique activating receptor and a type II transmembrane-anchored glycoprotein. It transmits signals through its transmembrane segment associating with adaptor protein DAP10. In the absence of DAP10, NKG2D is not able to be presented on cell surface and is retained in the cytoplasm (Diefenbach et al., 2002, Wu et al., 1999). Expression of NKG2D can be regulated by cytokines, for example, IL-15 and TNF-$\alpha$ can enhance NKG2D expression whereas TGF-$\beta$ causes its downregulation (Castriconi et al., 2003, Lee et al., 2004, Parasa et al., 2012). In humans, KIR3DL1 (CD158e1) and NKG2D are two representative signaling receptors expressed by $CD8^+$ T-lymphocytes, NK cells, NKT cells and $\gamma\delta$ $TCR^+$ T cells (Ferrini et al., 1994, Moretta et al., 1990, Patterson et al., 2008, Phillips et al., 1995, Wu et al., 1999). They can recognize MHC class I molecules and MHC class I-related proteins, respectively.

In this chapter, the activation levels (by measurement of CD69 and CD25 expression) of the three main classes of human killer cells, namely, $CD8^+$ T-lymphocytes, NKT-like

cells and NK cells in peripheral blood were investigated. Samples were obtained from four groups of participants including healthy non-smoking volunteers, current healthy smokers, current smokers with COPD and ex-smokers with COPD. To further detect the association between cell activation and their functional receptors in peripheral blood samples, we also examined the expression of the inhibitory receptor KIR3DL1 (CD158e1) and the activating receptor NKG2D in these four groups.

### 3.1.2 Material and methods

#### 3.1.2.1 Study population and procedures

The Nottingham Research Ethics Committee approved the study protocol (REC reference 04/Q2403/102) and written informed consent was obtained from all participants before entering the study. Thirty-two participants were included in both the peripheral blood activation and the KIR study. Participants were diagnosed as having COPD according to the ATS guidelines, were either current smokers with COPD (cuS-COPD) or ex-smokers with COPD (exS-COPD) and had accrued at least a 20-pack year smoking history. Healthy non-smokers (HNS) and current healthy smokers (HS), with an $FEV_1$ above 80% of predicted, were matched, as far as possible, for age, with participants with COPD; healthy smokers were also

matched for smoking history. Table 3.1.1 details the demographic and spirometric data of the participants in this study. Participants were excluded as previously stated ; specifically, they were all non-atopic (negative skin prick test response to common allergen extracts including grass pollen, house dust mite, cat dander and dog hair) and all COPD patients had been free of an acute exacerbation of COPD for at least 6 weeks preceding the study. In addition, none had received antibiotics or corticosteroids (oral and inhaled) over the same period.

### 3.1.2.2 Peripheral Blood Mononuclear Cell isolation

PBMCs were isolated from whole blood on a discontinuous histopaque density gradient (Sigma, Poole, UK). Briefly, 50ml of whole blood were diluted 2:1 in RPMI 1640 medium (Sigma, Poole, UK), were diluted 2:1 in RPMI 1640 medium (Sigma, Poole, UK), layered over histopaque and centrifuged for 22 minutes at 800g. The resultant mononuclear layer was removed and washed twice and resuspended. Cells were counted with a Hemocytometer using trypan blue.

**Table 3.1.1 Demographic and spirometric values of the studied groups**

| | Healthy non-smokers | Current healthy smokers | cuS-COPD | exS-COPD |
|---|---|---|---|---|
| **Participants** | 8 | 8 | 9 | 7 |
| **Age (years)** | 54 (47-66) | 53 (45-63) | 61 (45-77) | 62 (49-69) |
| **Gender (M/F)** | 4/4 | 3/5 | 3/6 | 3/4 |
| **Packs/yrs** | 0 | 35 (32-40) | 56 (30-77) | 36 (20-51) |
| **Smoking status (Current/Ex)** | 0 | 8/0 | 9/0 | 0/7 |
| **Chronic bronchitis (Yes/No)** | 0 | 3/5 | 6/3 | 5/2 |
| **$FEV_1$ (% pred)** | 105 (75-126) | 101 (85-114) | 41 (30-61) | 58 (52-66) |
| **$FEV_1$/FVC (%)** | 104 (67-120) | 89 (66-101) | 48 (29-53) | 51 (36-65) |
| **Δ$FEV_1$ post bronch** | 2.2 (1.9-3.0) | 2.1 (1.7-2.9) | 1.6 (1.1-2.4) | 1.1 (0.6-1.5) |
| **BMI ($kg/m^2$)** | 22.8 (18.7-26.2) | 24.7 (19.2-31.6) | 23.2 (18.3-30.8) | 21.5 (19.6-23.1) |
| **Inhaled GCS (on/off)** | N/A | N/A | 3/6 | 4/3 |
| **MRC dyspnoea scale** | N/A | N/A | 3 (2-4) | 2 (1-4) |
| **Distance walked in 6 min (m)** | N/A | N/A | 308 (50-431) | 277 (140-456) |
| **BODE Index** | N/A | N/A | 5 (2-7) | 5 (1-6) |

Results are expressed as median with range in brackets.

exS-COPD, ex-smokers with COPD; cuS-COPD, current smokers with COPD; $FEV_1$, forced expiratory volume in 1 second; pre, predicted value; FVC, forced vital capacity; BMI, body mass index; GCS, corticosteroid; MRC, medical research council; BODE, **B**MI, airflow **O**bstruction, **D**yspnoea and **E**xercise capacity.

### 3.1.2.3 Flow cytometric analysis

Cells were stained for flow cytometry as previously described . Briefly, cells were fixed in 3% formaldehyde in isotonic azide free solution (Beckman Coulter, Luton, UK) and washed before labelled antibodies (Table 3.1.2) were added at the recommended concentration. The cells were incubated in the dark and excess antibody removed. Flow cytometric analysis of antibody labelled cells was performed using an EPICS Altra (Beckman Coulter, Luton, UK). Fifty thousand live-gated events were collected for each sample and isotype matched antibodies were used to determine binding specificity. Data were analysed using WEASEL version 2.3 (WEHI). Necrotic cells were excluded from analysis according to their forward and side scatter characteristics.

### 3.1.2.4 Statistical analysis

The statistical analysis was performed with Prism software, version 4.0c (GraphPad). Normality was detected using the Kolmogorov–Smirnov test. All data are expressed as median and range. Differences between the four groups of participants were tested using the non-parametric Kruskal-Wallis test with *post hoc* pairwise comparisons made by the Dunn's Multiple Comparison test to determine which pair was

statistically significantly different. P values of less than 0.05 were considered to indicate statistical significance.

**Table 3.1.2 Antibodies used for flow cytometry**

| Antigen | Fluorochrome | Isotype | Clone | Source |
|---|---|---|---|---|
| CD3 | ECD<br>PC7 | Mouse IgG1 | UCHT1 | Beckman Coulter, Luton, UK |
| CD8 | PC5<br>APC | Mouse IgG1 | B9.11 | Beckman Coulter, Luton, UK |
| CD8 | ECD | Mouse IgG1 | SFCl21Thy2 D3 | Beckman Coulter, Luton, UK |
| CD25 | FITC<br>PE | Mouse IgG2a | B1.49.9 | Beckman Coulter, Luton, UK |
| CD56 | PE<br>PC5<br>PC7 | Mouse IgG1 | N901 | Beckman Coulter, Luton, UK |
| CD69 | ECD<br>PC5 | Mouse IgG2b | TP1.55.3 | Beckman Coulter, Luton, UK |
| KIR3DL1 (CD158e1) | FITC | Mouse IgG1 | DX9 | Biolegend, San Diego, CA, USA |
| NKG2D (CD314) | PE | Mouse IgG1 | ON72 | Beckman Coulter, Luton, UK |

### 3.1.3 Results

#### 3.1.3.1 Demographic and medical characteristics of COPD participants, current healthy smokers and healthy controls

There were no statistical difference between groups in terms of age (Figure 3.1.1.A) or pack years smoked (Figure 3.1.1.B) between ex-smokers with COPD, current smokers with COPD and current healthy smokers. Furthermore, there was no statistical difference in the inhaled corticosteroid use between ex–smokers with COPD and current smokers with COPD.

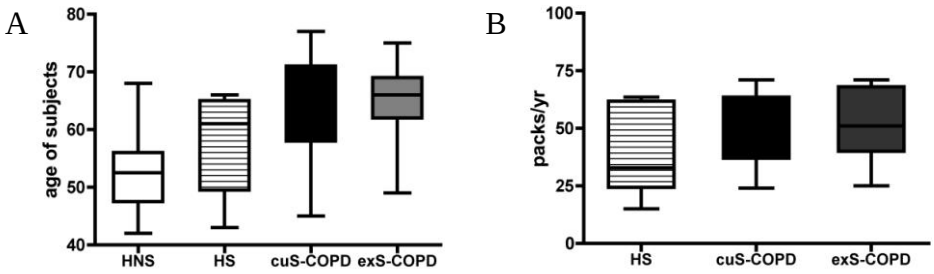



**Figure 3.1.1 Comparison of ages and pack year smoked between interested groups.** There were statistical differences in terms of (A) ages or (B) pack year smoked between healthy non-smokers (HNS, n=8), current healthy smokers (HS, n=9), current smokers with COPD (cuS-COPD, n=9) and ex-smokers with COPD (exS-COPD, n=7).

### 3.1.3.2 Killer cell activation in peripheral blood

All individuals had similar total mononuclear cell numbers, which were within the normal range (data not shown). Representative flow analysis dot plots of Forward Scatter (FS) vs Side Scatter (SS) for identification of live and dead cells (Figure 3.1.2.A), along with dot plots for identification of $CD8^+$ T-lymphocytes ($CD3^+CD8^+$), $CD4^+$ T-lymphocytes ($CD3^+CD4^+$) (Figure 3.1.2.B), NKT-like cells ($CD3^+CD56^+$), and NK cells ($CD3^-CD56^+$) (Figure 3.1.2C) are shown. In addition, exemplar dot plots for CD69 and CD25 staining of these gated four cell types from peripheral blood in the four groups are shown in Figure 3.1.3. Cells are designated activated when they express one or both of the activation markers (i.e., CD69 positive, CD69/CD25 double positive or CD25 positive).

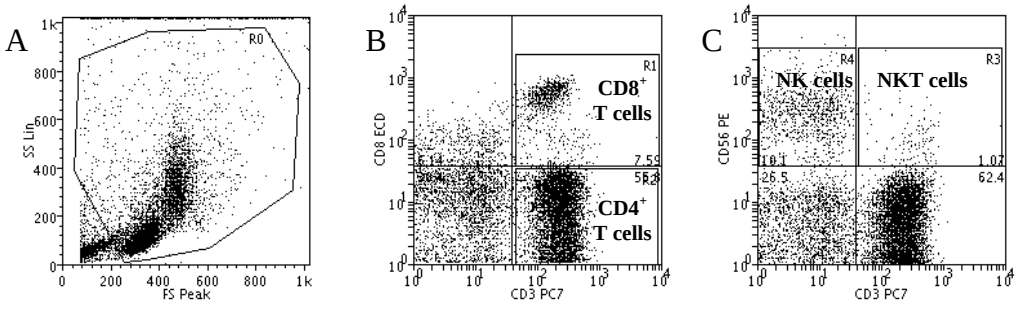
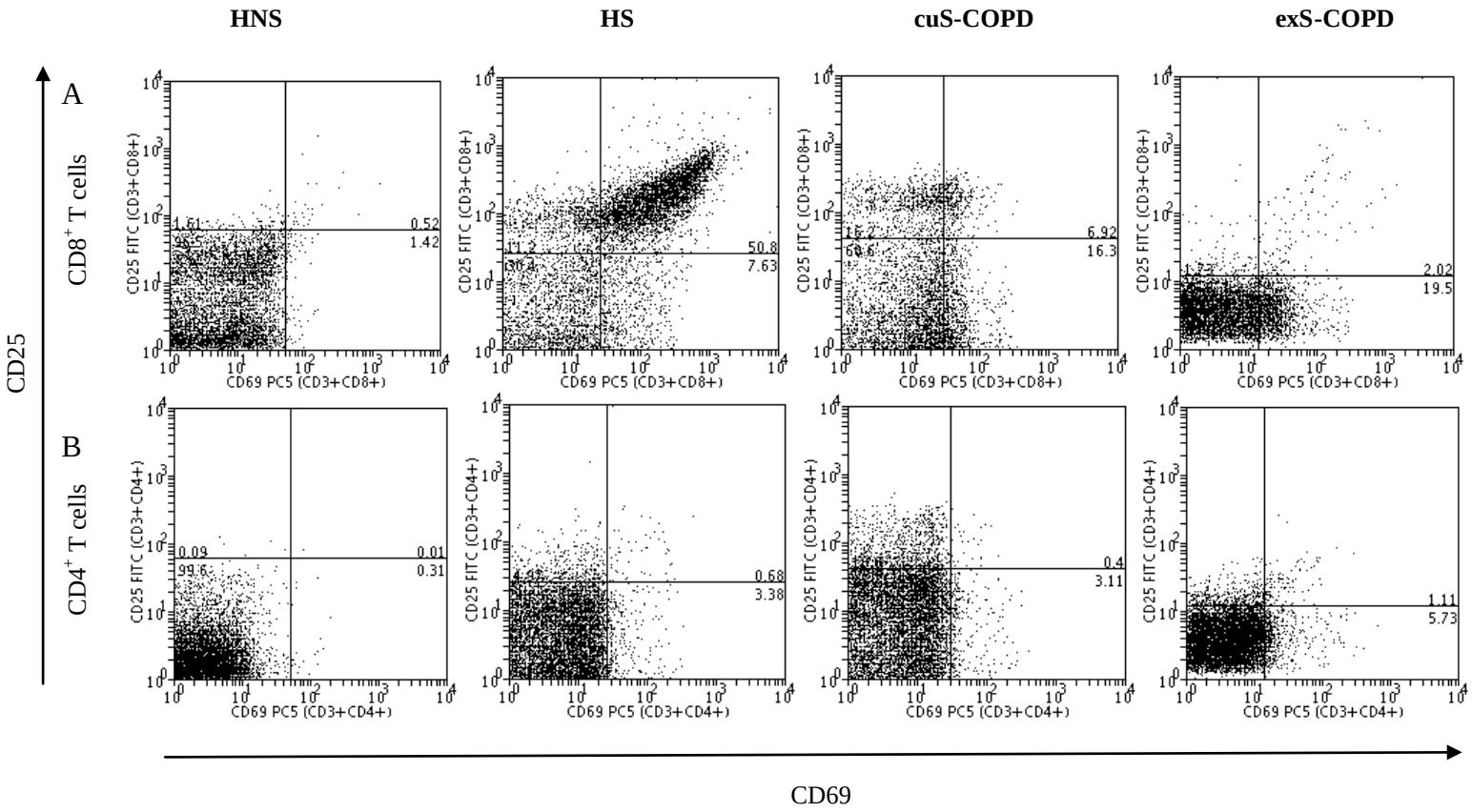



**Figure 3.1.2 Representative dot plots gating for live/dead cells, identification of cell populations from current smokers with COPD.** (A) Forward Scatter (FS) vs Side Scatter (SS) plot for identification of live and dead cells; (B) and (C), Identification of $CD8^+$ T cells ($CD3^+CD8^+$), $CD4^+$ T cells ($CD3^+CD4^+$), NKT-like cells ($CD3^+CD56^+$) and NK cells ($CD3^-CD56^+$).


HNS
HS
cuS-COPD
exS-COPD
A
CD8+ T cells
B
CD4+ T cells
CD25
CD69
CD25 FITC (CD3+CD8+)
CD69 PC5 (CD3+CD8+)
CD25 FITC (CD3+CD4+)
CD69 PC5 (CD3+CD4+)
1.61
0.52
1.42
11.2
50.8
7.63
15.2
6.92
16.3
1.72
2.02
19.5
0.09
99.6
0.01
0.31
4.6
0.68
3.38
0.4
3.11
1.11
5.73

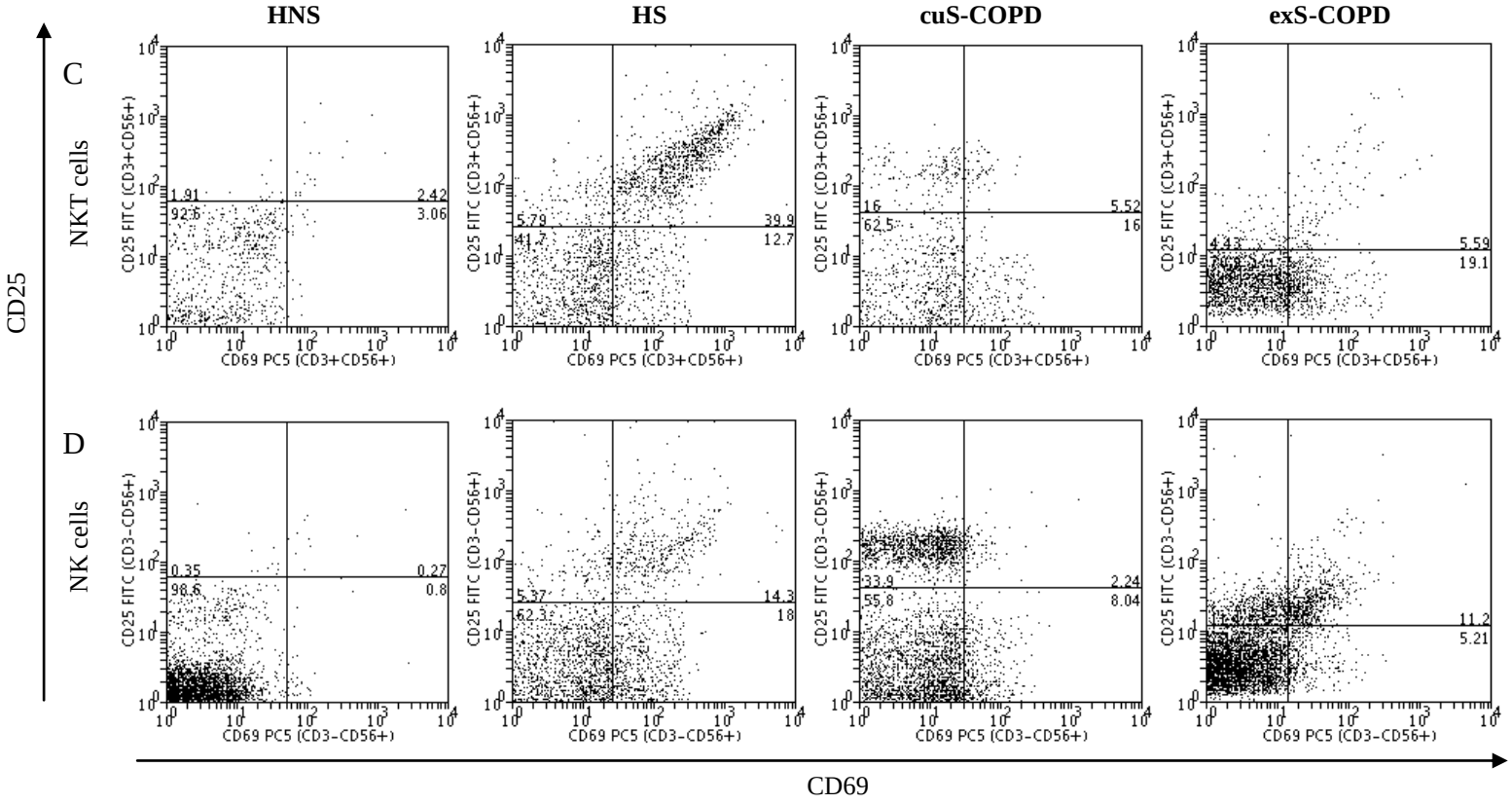



**Figure 3.1.3 Representative dot plots for activation of interested cell types from four groups including healthy non-smokers (HNS), current healthy smokers (HS), current smokers with COPD (cuS-COPD) and ex-smokers with COPD (exS-COPD).** Activation is designated by expressing either CD69 alone (lower right quadrant), CD69 and CD25 (upper right quadrant) or CD25 alone (upper left quadrant). (A) $CD8^+$ T cells; (B) $CD4^+$ T cells; (C) NKT-like cells; and (D) NK cells.

Significantly more $CD8^+$ T-lymphocytes from current healthy smokers (41.96%, 27.2-72.8; $p<0.001$), current smokers with COPD (34.3%, 13.8-56.2; $p<0.01$), and ex-smokers with COPD (23%, 11.01-48.4; $p<0.05$) were activated *ex vivo* compared to healthy non-smokers (3.78%, 2.5-9.3) (Figure 3.1.4.A). Analyses of individual markers (i.e., CD69 positive/CD25 negative; CD69 negative/CD25 positive; or CD69 positive/CD25 positive) were not different between groups or cell types (data not shown).

NKT-like cells showed a similar pattern to $CD8^+$ T-lymphocytes, namely significantly more *ex vivo* activation from both current healthy smokers (40.05%, 17.7-58.7; $p<0.001$) and current smokers with COPD (33.04%, 15.7-48.8; $p<0.01$) compared to healthy non-smokers (7.3%, 4.26-16.82) (Figure 3.1.4.C). Also, no significant differences in NKT-like cell activation were observed between ex-smokers (27.05%, 12.4-48.1) and healthy non-smokers (7.3%, 4.26-16.82; $p>0.05$), current healthy smokers (40.05%, 17.7-58.7; $p>0.05$), or current smokers with COPD (33.04%, 15.7-48.8; $p>0.05$).

NK ($CD56^+CD3^-$) cells from both current healthy smokers (39.55%, 14.78-50.1; $p<0.05$) and current smokers with COPD (44.18%, 14.2-72.8; $p<0.01$) were significantly more

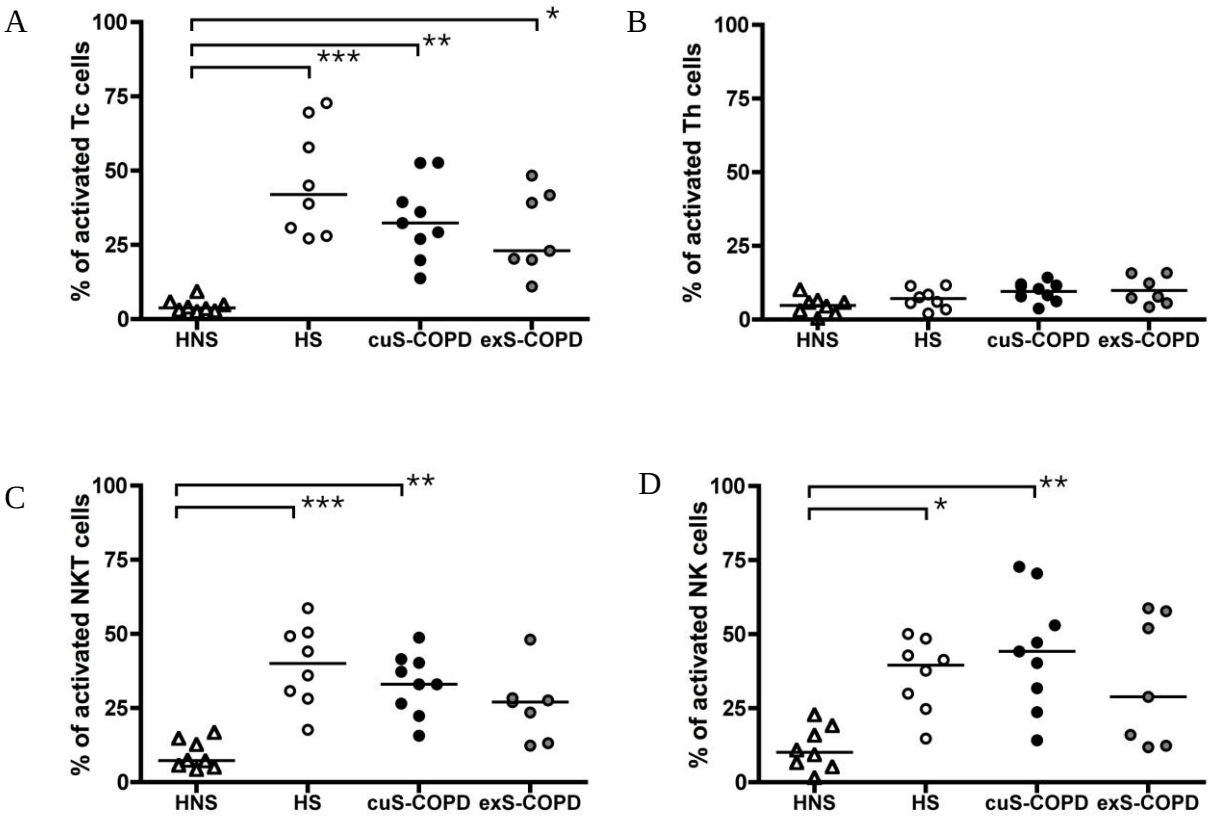



**Figure 3.1.4 Activation of killer cell types from peripheral blood *ex vivo* of four groups.** Activation of $CD8^+$ T cells, $CD4^+$ T cells, NKT-like cells and NK cells were analysed. There was a significant increase in level of activation of $CD8^+$ T cell (panel A), NKT-like cells (panel C), and NK cells (panel D) from current healthy smokers (HS, open circles) and current smokers with COPD (cuS-COPD, black solid circles). Activation of NK cells from ex-smokers with COPD (exS-COPD, grey solid circles) was also significantly increased compared to healthy non-smokers (HNS, open triangle). There was no statistical difference in activation of $CD4^+$ T cells (panel B) between groups. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. HNS (n=8), HS (n=8), cuS-COPD (n=9), and exS-COPD (n=7).

activated *ex vivo* than those from healthy non-smokers (10.1%, 1.5-22.8) (Figure 3.1.4.D). There were no significant differences in NK cell activation between ex-smokers with COPD (28.9%, 11.91-58.8; $p>0.05$) and other groups of interest.

Interestingly, few $CD4^+$ T lymphocytes were activated in peripheral blood from four groups (Figure 3.1.4.B) and there were no statistical differences between groups (all p value >0.05), namely, healthy non-smokers (5.17%, 0.4-10.16), current healthy smokers (6.87%, 2.16-11.7), current smokers with COPD (10.46%, 3.81-14.31) and ex-smokers with COPD (7.81%, 4.24-15.89).

### 3.1.3.3 Expression of CD25 on $CD4^+$ T cells

Although activation of $CD4^+$ T lymphocytes was lower than other cell types of interest (Figure 3.1.4.B), activation markers including CD69 and CD25 were still detectable on their cell surface (Figure 3.1.3.B). Regulatory T (Treg) cells, as a subset of $CD4^+$ T cells, also express CD25. Therefore, we hypothesised that those $CD4^+CD25^+$ T cells could be Treg cells. To examine this further we generated dot plots of Forward Scatter (FS) vs Side Scatter (SS) that showed, unlike other activated cell types expressing CD25 that were larger and

more granular (Figure 3.1.5.A, C and D), those $CD4^+$ $CD25^+$ T cells were primarily small and less granular and thus quite probably inactivated lymphocytes (Figure 3.1.5.B). These observations were seen in all groups of interest.

### 3.1.3.4 Correlation of cell activation and number of cigarettes being smoked per day

In view of our findings that current smoking increased numbers of activated Tc, NKT and NK cells, we looked for correlations between the number of cigarettes currently being smoked per day and level of *ex vivo* cell activation for these three cell types from both current healthy smokers and current smokers with COPD (Figure 3.1.6). Cells expressing CD69 or CD25 alone, and expressing both activation markers were considered as activated cells in each cell type. In current smokers with COPD, NK cells showed a significant positive correlation between the proportion of *ex vivo* activation and number of cigarettes smoked ($r=0.7615$, $p=0.0214$) (Figures 3.1.6.C). No such correlation was observed for $CD8^+$ T cells ($r=0.2427$, $p=0.5206$) (Figure 3.1.6.A), NKT-like cells ($r=0.0753$, $p=0.8432$) (Figure 3.1.6.B). Also, there was no significant correlation between the proportion of ex vivo activation and number of cigarettes smoked in these cell types from current healthy smokers (Figure 3.1.6).

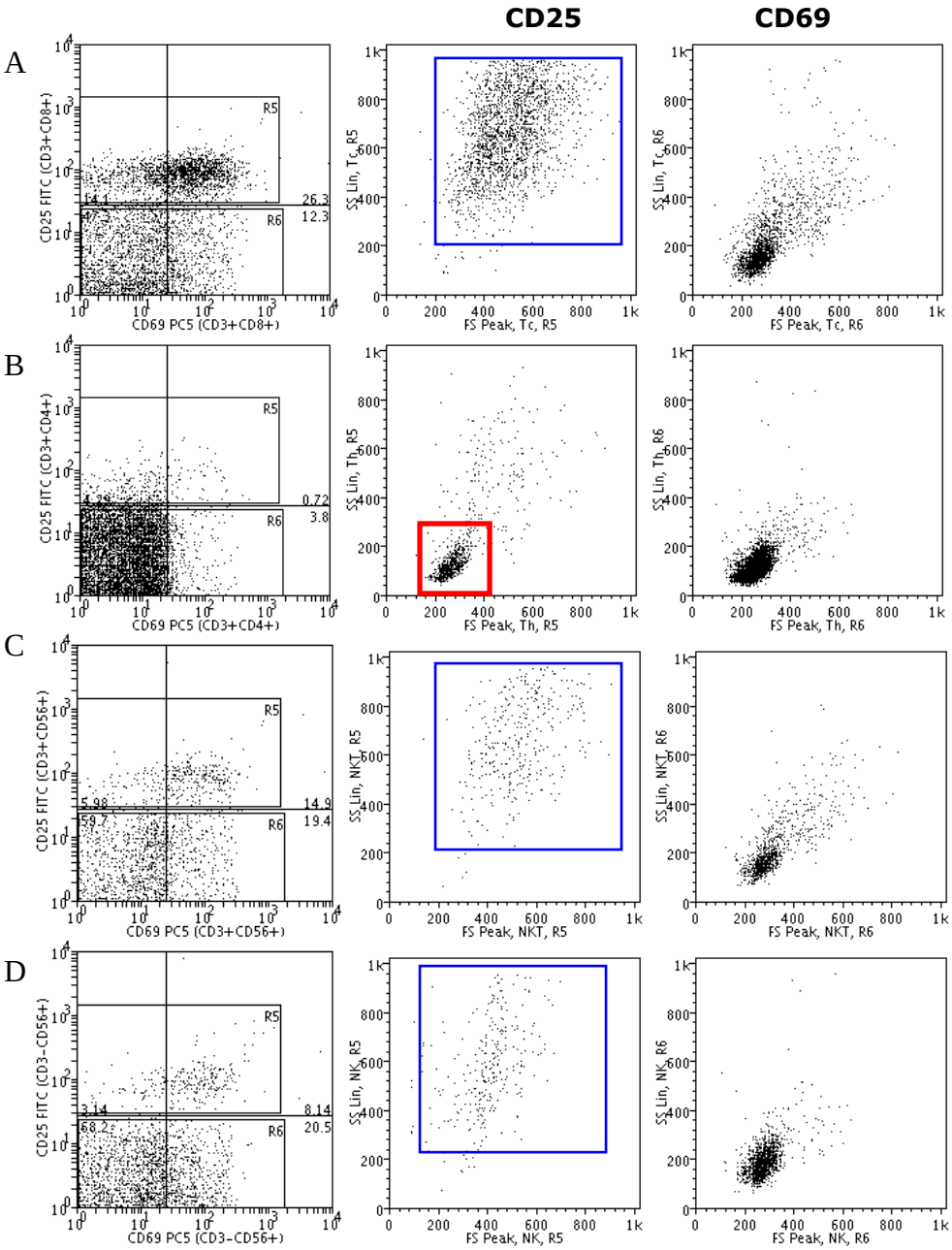
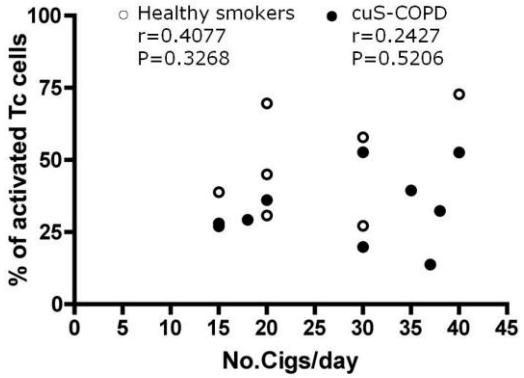
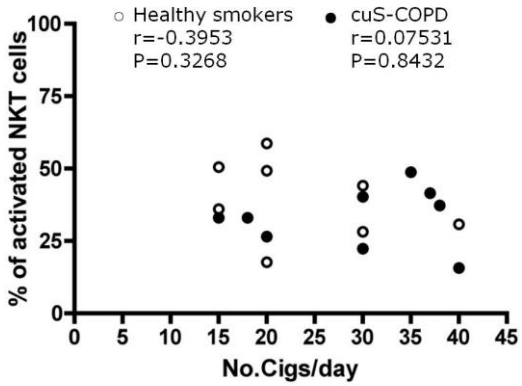



**Figure 3.1.5 Representative dot plots for gating cell types that express CD25 from current smokers with COPD.** CD69 and CD25 are cell surface markers of early and later stages of lymphocyte activation, respectively. Unlike other cell types of interest, $CD4^+$ T cells expressing CD25 was mainly lymphocytes (red square) but not activated cells (blue square). Regulatory T (Treg) cells are a subset of $CD4^+$ T cells expressing CD25, which indicated that those $CD4^+CD25^+$ T cells might be Treg cells. (A) $CD8^+$ T cell; (B) $CD4^+$ T cells; (C) NKT-like cells; and (D) NK cells.

A




B




C

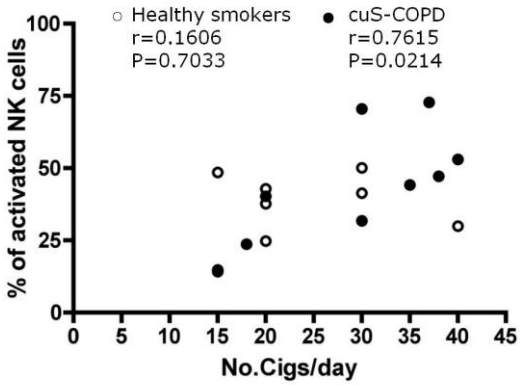


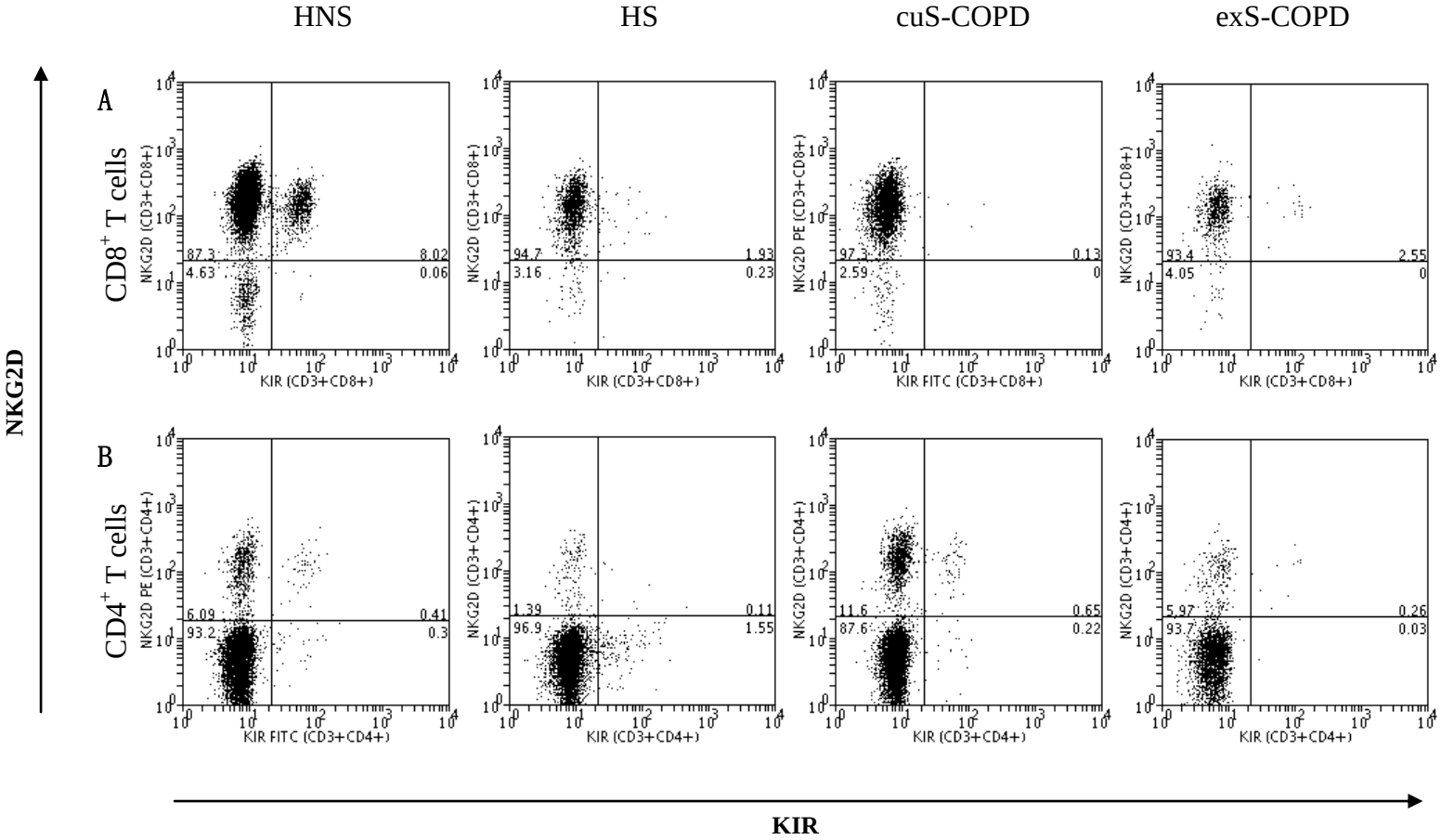

**Figure 3.1.6 Correlation of the proportion of cells activated and number of cigarettes currently smoked per day in current current healthy smokers (HS) and current smokers with COPD (cuS-COPD).** Analysis was performed on peripheral blood $CD8^+$ T lymphocytes (Panel A), NKT-like cells (Panel B) and NK cells (Panel C). A significant correlation was observed in NK cells from current smokers with COPD. HS, n=8; cuS-COPD, n=9.

### 3.1.3.5 KIR (CD158e1) and NKG2D expression in peripheral blood

The balance between activation receptors and inhibitory receptors expressed by cells is one of crucial factors orchestrating cell activation. Expression of both the inhibitory KIR (CD158e1) and the activating receptor NKG2D by killer cells, including $CD8^+$ T cells (Figure 3.1.7.A), $CD4^+$ T cells (Figure 3.1.7.B), NKT cells (Figure 3.1.7.C) and NK cells (Figure 3.1.7.D), were measured by flow cytometry. Representative dot plots of KIR and NKG2D expression from healthy non-smokers (HNS); current healthy smokers (HS); current smokers with COPD (cuS-COPD); and ex-smokers with COPD (exS-COPD) are shown in Figure 3.1.7.

$CD8^+$ T-lymphocytes showed a significantly lower proportion of cells from healthy smokers (0.83%, 0.12-2.16; $p < 0.001$) and current smokers with COPD (0.66%, 0.13-3.97; $p < 0.001$) expressed CD158e1 on the cell surface compared to healthy non-smokers (9.91%, 4.78-16.6) (Figure 3.1.8.A).

The proportion of NKT-like cells expressing surface CD158e1 was also significantly lower in current healthy smokers (2.14%, 0.24-7.92; $p < 0.001$) and current smokers with COPD (2.24%, 1.58-6.77; $p < 0.001$) compared to healthy


HNS
HS
cuS-COPD
exS-COPD
A
CD8+ T cells
NKG2D (CD3+CD8+)
KIR (CD3+CD8+)
87.3
8.02
4.63
0.06
94.7
1.93
3.16
0.23
97.3
0.13
2.59
0
93.4
2.55
4.05
0
B
CD4+ T cells
NKG2D PE (CD3+CD4+)
KIR FITC (CD3+CD4+)
KIR (CD3+CD4+)
6.09
0.41
93.2
0.3
1.39
0.11
96.9
1.55
11.6
0.65
87.6
0.22
5.97
0.26
93.7
0.03
NKG2D
KIR

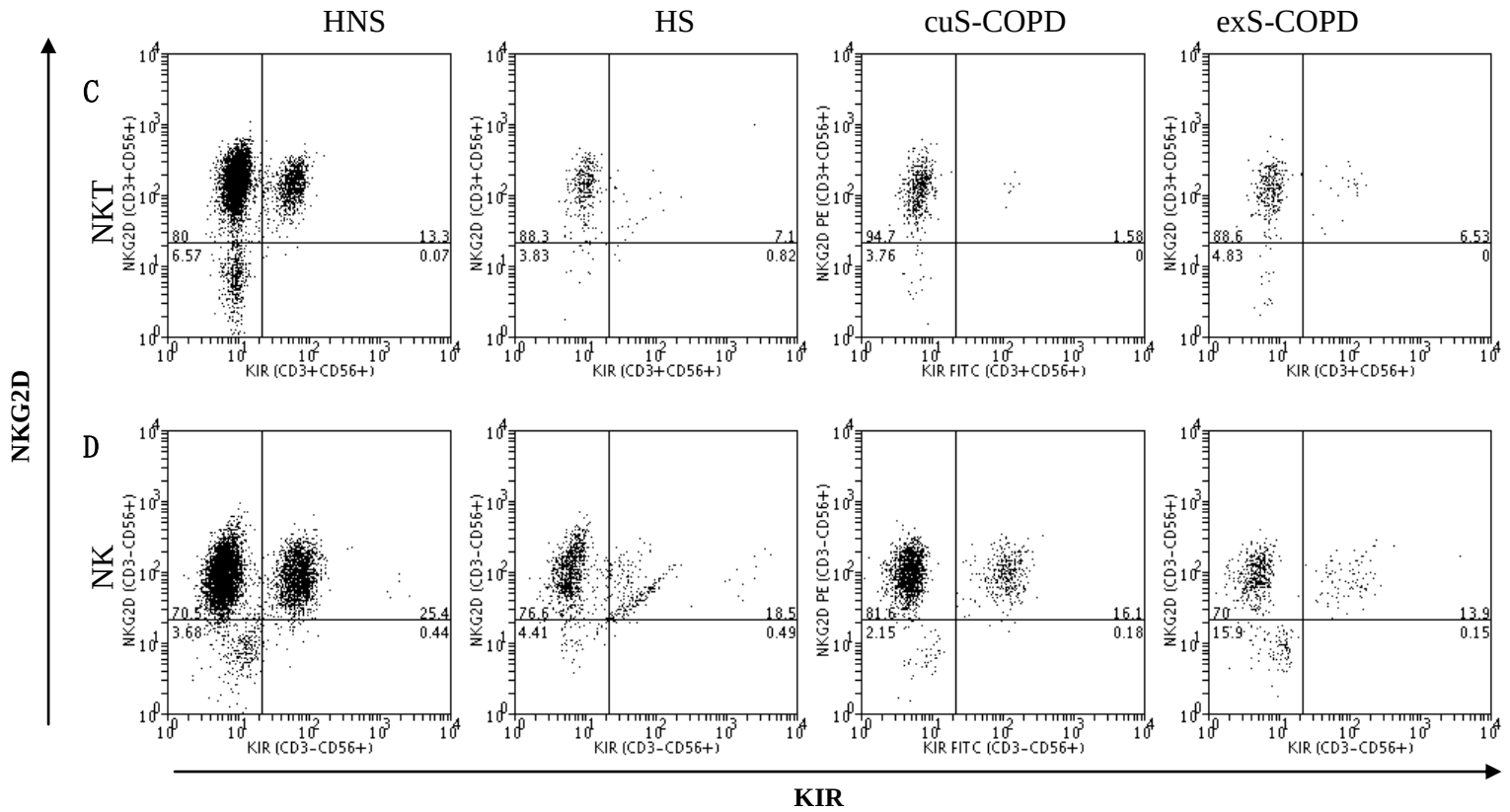



**Figure 3.1.7 Representative dot plots for KIR (CD158e1) and NKG2D expression of cell types of interest from four groups including healthy non-smokers (HNS), current healthy smokers (HS), current smokers with COPD (cuS-COPD) and ex-smokers with COPD (exS-COPD).** NKG2D was highly expressed by $CD8^+$ T cells, NKT-like cells and NK cells but not $CD4^+$ T cells. (A) $CD8^+$ T cells; (B) $CD4^+$ T cells; (C) NKT-like cells; and (D) NK cells.

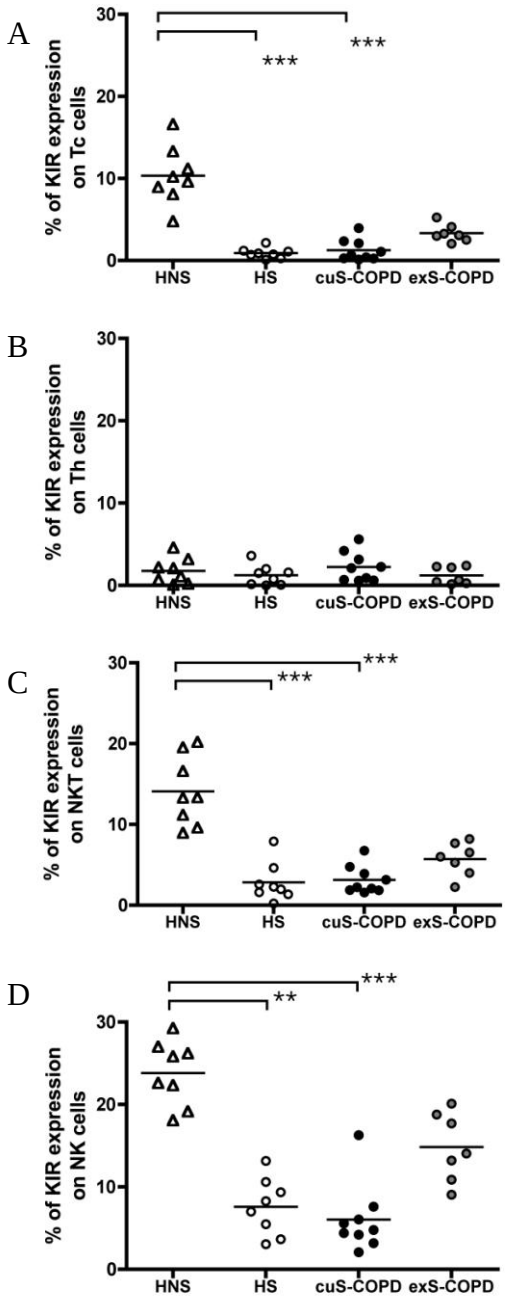



**Figure 3.1.8 KIR (CD158e1) expression of cell types of interest from peripheral blood *ex vivo* of four groups.** Levels of KIR expression by $CD8^+$ T cell (panel A), NKT-like cells (panel C), and NK cells (panel D) were all significantly reduced in current healthy smokers (HS, open circles) and current smokers with COPD (cuS-COPD, solid circles) compared to healthy non-smokers (HNS, open triangles). There was no significant difference in the KIR expression between ex-smokers with COPD (exS-COPD, grey solid circles) and other groups. There was also no statistical difference in the levels of KIR expression on $CD4^+$ T cells (panel B) between groups. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. HNS (n=8), HS (n=8), cuS-COPD (n=9), and exS-COPD (n=7).

non- smokers (13.34%, 8.97-20.2) (Figure 3.1.8.C).

Analysis of NK cells showed the proportion of these cells expressing surface CD158e1 was also significantly lower in current healthy smokers (7.7%, 3.06-13.17; $p < 0.01$) and current smokers with COPD (4.79%, 2.08-16.28; $p < 0.001$) compared to healthy non-smokers (24.22%, 18.1-29.26) (Figure 3.1.8.D).

No differences in proportions of $CD4^+$ T-lymphocytes (in all cases median levels <2.1%) expressing CD158e1 were shown between the four groups (Figure 3.1.8.B).

Interestingly, proportions of $CD4^+$ T lymphocytes expressing NKG2D were significantly lower (in all cases median levels <9.0%) compared to proportions of $CD8^+$ T-lymphocytes, NKT-like cells and NK cells (in all cases over 95%) in each group. Meanwhile, there were no statistical differences on the proportion of each cell type expressing NKG2D between four groups (Figure 3.1.9).

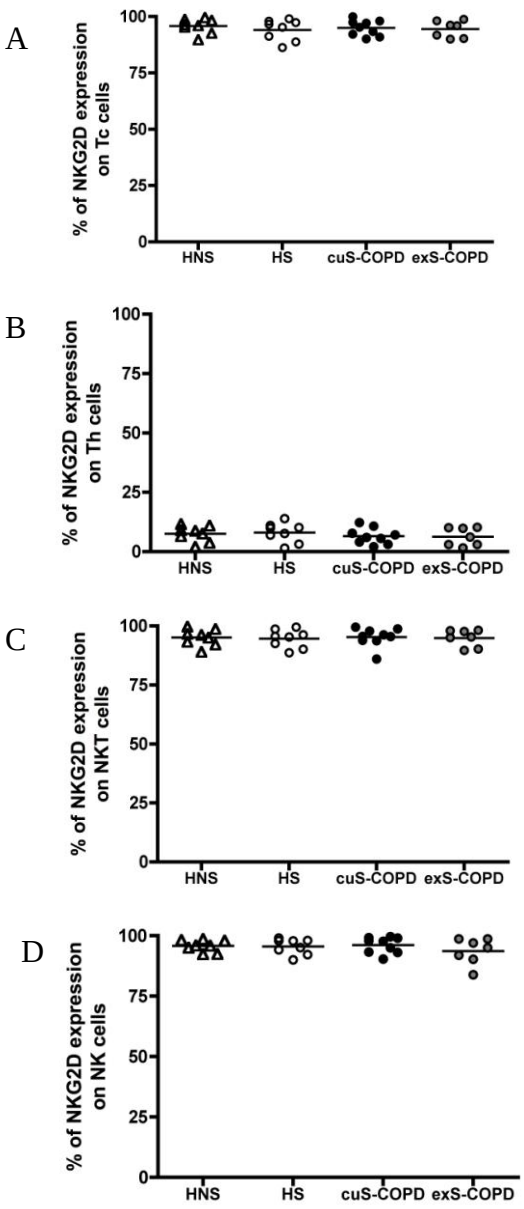



**Figure 3.1.9 Proportions of NKG2D expression on cell types of interest from peripheral blood *ex vivo* of four groups.** NKG2D expression by $CD8^+$ T cells (Tc, panel A), $CD4^+$ T cells (Th, panel B), NKT-like (NKT, panel C) cells and NK cells (NK, panel D) were analysed. There were no significant differences on the proportion of NKG2D expression by each cell type from four groups, including healthy non-smokers (n=8), current healthy smokers (n=8), current smokers with COPD (cuS-COPD, n=9), and ex-smokers with COPD (exS-COPD, n=7). All p value >0.05.

### 3.1.4 Discussion

Here we report, for the first time, that current smoking *per se* is associated with a significantly higher proportion of peripheral blood cytotoxic cells that are activated *ex vivo* and this is positively correlated with the number of cigarettes currently smoked per day.

We and others have previously demonstrated the potential involvement of the three main classes of human killer cells, namely, $CD8^+$ T-lymphocytes, NKT-like cells and NK cells in COPD (Paats et al., 2012, Roos-Engstrand et al., 2009, Roos-Engstrand et al., 2010, Urbanowicz et al., 2009). These killer cells play key roles in inflammatory responses and activation of these cells can cause the production of inflammatory cytokines and chemokines, such as TNF-$\alpha$ and IL-8, that can induce pathological features of COPD and lung tissue damage (Barnes, 2004c). Therefore, we hypothesized that killer cells are more active in COPD patients. Here we examined the *ex vivo* activation phenotype of these cells in the peripheral blood. In peripheral blood, current smokers with or without COPD, but not ex-smokers with COPD, have the highest proportion of activated $CD8^+$ T-lymphocytes, NKT-like cells and NK cells compared with healthy non-smokers and ex-smokers with COPD. This suggests that smoking *per se* affects systemic

killer cell activation. We thus examined the correlation between current smoking profiles and cell activation levels. Here we show that NK cell activation is positively correlated with the number of cigarettes currently smoked in COPD patients. No correlations between cell activation and lung function ($FEV_1$ percentage of predicted) were found (data not shown). These data suggest that in peripheral blood, cell activation levels are more related to current smoking habit rather than disease. A previous study examining the effect of smoking cessation has shown in BAL fluid that $CD8^+$ T-lymphocytes from both smokers with normal lung function and COPD participants expressed high levels of CD69 and CD25 activation markers (Roos-Engstrand et al., 2009). Sputum T-lymphocytes have also been shown to express higher levels of CD103 and CD69 than blood lymphocytes suggesting higher numbers of activated intraepithelial phenotype T-lymphocytes in the lung (Leckie et al., 2003). Furthermore, activated $CD8^+$ T cells secrete more CXCR3 that lead to the accumulation of $CD8^+$ T cells to the infected tissue and organs, which may explain why $CD8^+$ T cells are predominant increased in COPD but not $CD4^+$ T cells. Interestingly, here we also show few $CD4^+$ T cells are activated in peripheral blood from the four groups. We further show that those $CD4^+CD25^+$ T cells may be regulatory T cells (not activated cells) by dot plot of

Forward Scatter (FS) vs Side Scatter (SS). To confirm this observation further studies are required. Although two studies have found highly activated Th1 cells in the lung of ex-smokers with COPD and in the smalll airway wall of smokers with severe COPD by detecting their chemokine and cytokine secretion, those samples are actually from COPD patients with emphysema or co-suffering from cancer (Turato et al., 2002).

One of the crucial factors determining cell activation is the signaling balance between activating receptors and inhibitory receptors. To investigate a possible mechanism for differences in activation states observed in peripheral blood, we compared expression of the inhibitory receptor KIR CD158e1 and the activating receptor NKG2D. Interestingly, in this study, the expression levels of the inhibitory KIR CD158e1 were lower in peripheral blood of current smokers with or without COPD compared to healthy non-smokers, which was the opposite to the activation levels of these killer cells including $CD8^+$ T cells, NKT-like cells and NK cells. Similar study supports this finding. CD94 is another inhibitory receptor expressed by NKT-like cells and NK cells (Lazetic et al., 1996). Decreased expression of CD94 on both NKT-like cells and NK cells were detected in PBMCs from COPD patients compared to healthy volunteers (Hodge et al., 2012). However, they did not detect any

significant differences on the cell activation levels of NKT-like and NK cells (only CD69 expression) between groups. Therefore, our findings provided the first demonstration of association of cell activation and KIR expression. We also show that activating receptor NKG2D are highly expressed by $CD8^+$ T cells, NKT-like cells and NK cells but not $CD4^+$ T cells, which is in accordance with previous studies that demonstrate NKG2D receptors are constitutively expressed and almost exclusively on cytotoxic cells including $CD8^+$ T cells, NKT cells, NK cells and $\gamma\delta$ T cells (Maasho et al., 2005). There are no significant differences of NKG2D expression on $CD8^+$ T cells, $CD4^+$ T cells, NKT-like cells and NK cells respectively, in the four groups. This further supports the proposal of increased peripheral blood killer cell activity in smokers due to the increased ratio of activating to inhibitory receptors.

The present study does have some limitations that deserve comment. Firstly, we used CD69 and CD25 as activation markers. Although other markers exist, these are generally accepted as representing cell activation (Marquez et al., 2010, McDonald et al., 2010). Secondly, CD25 is commonly used as a marker for T regulatory cells (Lane et al., 2010). To further confirm whether these $CD4^+CD25^+$ T cells are Treg cell, FOXP3 can be used as a good marker for Treg cell indentification.

Also, fluorphore FITC is not particularly bright, they photobleach rapidly and are self-quenching at high concentration. Therefore, other fluorophore, such as the AlexaFluors, may be applied in future study to gain high quality analysis. Thirdly, there are many kinds of inhibitory and activating receptors expressed on these cells but due to limited cell numbers we analysed two representative receptors that give an indication of the killing function of these cells. Fourthly, it should be noted that the majority of participants with COPD were on inhaled corticosteroids. However, the two participants who were not on inhaled corticosteroids in this group showed the same results in terms of activation and KIR expression as those on inhaled corticosteroids.

Of the note, our colleague also detected, *ex vivo*, the activation levels of killer cells in sputum samples from COPD patients (data shown in the publication paper). Unlike systemic activation, lung activation is due specifically to COPD, irrespective of current smoking status.

In summary, we have shown for the first time the activation levels of killer cells from peripheral blood of COPD patients. Also, the systemic activation is in response to smoking *per se*. We further demonstrate expression levels of inhibitory

receptor KIR (CD158e) in COPD groups. These findings indicate that activation of killer cells in COPD participants may play a role in the pathogenesis of COPD.

## 3.2 Effects of cigarette smoke extract on killer cell activation in COPD

### 3.2.1 Introduction

***Cigarette smoke extract (CSE)***

Cigarette smoke has been identified as the main risk factor in COPD development. Previous studies have demonstrated that cigarette smoke affects structural and functional changes in the respiratory ciliary epithelium (Cantin et al., 2006, Mio et al., 1997, Wyatt et al., 1999), lung surfactant protein and immune cells (Mohan, 2002a), such as lymphoctyes, alveolar macrophages, dendritic cells, neutrophils and NK cells. Therefore, accumulating data suggested that chronic inhalation of cigarette smoke alters a wide range of immunologic function, including both innate and adaptive immune responses.

The smoke from a cigarette contains thousands chemicals which have various toxic, mutagenic and carcinogenic activity (Hoffmann and Wynder, 1986, Stedman, 1968). The concentrations of chemical ingredients also vary widely in the different cigarette brands. Studies show that various components of cigarette smoke can affect the immune

response in different cell types (Lee et al., 2007b, McCue et al., 2000, Ouyang et al., 2000). Two major components of smoke are nicotine and tar (Hoffmann and Wynder, 1986, Stedman, 1968). Previous studies have shown that nicotine is tightly associated with increased risk of cardiovascular disease and cancer, particularly of lungs. Tar is an irritant. It enters the respiratory system as an aerosol of minute droplets that are deposited in the respiratory system causing excessive mucus production. Tar also contains carcinogens that combine with the affected mucus membranes. It causes lung cancer, chronic bronchitis and emphysema. Holt *et al* has investigated that cigarette smoke containing high levels of tar and nicotine is more immunosuppressive than the smoke from low-tar low-nicotine cigarettes (Holt et al., 1976). However, effects of cigarette smoke on the immune system are biphasic, which is mainly determined by the components and duration of exposure. For example, unlike nicotine and tar, tobacco glycoprotein and metals present in cigarette smoke are generally immunostimulatory (Brooks et al., 1990, Francus et al., 1992)..

Immunological features of cigarette smoke in COPD have been explored in human studies. *In vitro* activation of T cells and expression of granzyme B were significantly suppressed by

cigarette smoke, which resulted in altered immune response of the lymphocytes (Glader et al., 2006a). Skold *et al* have demonstrated that exposure to cigarette smoke altered the phenotype of human alveolar macrophages (Skold et al., 1996). Recently, effects of cigarette smoke on DCs in COPD have been more explored. *In vitro*, the effects of cigarette smoke on DCs were dose-dependent. Production of cytokines such as IL-12 and IL-10 during DCs maturation process can influence DC induction of a Th1 or Th2 immune response. In addition to expressing high levels of antigen-presenting molecules and costimulatory molecules, mature DCs release large amounts of IL-12 that can stimulate a Th1 immune response. Release of IL-10, however, blocks the DC maturation process by interfering with up-regulation of costimulatory molecules and production of IL-12, subsequently limiting the ability of DCs to initiate a Th1 response (Corinti et al., 2001, De Smedt et al., 1997). Early studies have shown that Low doses of nicotine enhanced human monocyte–derived DC maturation and their capacity to secrete IL-12 (Aicher et al., 2003). On the contrary, higher doses of nicotine treated DCs had low endocytic and phagocytic function, produced less IL-12 and more Th2 cytokine IL-10 that promoted DCs priming Th2 cell differentiation more efficiently (Nouri-Shirazi and Guinet, 2003,

Nouri-Shirazi and Guinet, 2006). Similar results were also shown in Vassallo *et al* study. LPS-induced DC maturation was suppressed by cigarette smoke, while the secretion of IL-10 and prostaglandin E2 from these DCs were enhanced (Vassallo et al., 2005). Moreover, cigarette smoke impaired the production of TNF-$\alpha$, IL-6 and IFN-$\alpha$, whereas augmented the IL-8 secretion by plasmacytoid dendritic cells in response to Toll-like receptor (TLR)-9 (Mortaz et al., 2009). Studies on the effects of cigarette smoke on NK cell properties showed controversial findings. Some groups found that cigarette smoke impaired NK cell cytotoxic activities (Takeuchi et al., 1988), whereas others showed that their killer functions were increased or not affected (Meliska et al., 1995, Newman et al., 1991). However, these findings were generated from different disease models. Co-activation of NK cells by cigarette smoke and NK cell inducers have been established in Mian *et al* studies. Cigarette smoke greatly impaired the production of IFN-$\gamma$ and TNF-$\alpha$ by NK cells upon polyinosinic:polycytidylic acid (poly I:C) activation. The cytotoxic activities and perforin expression of poly I:C activated NK cells were also attenuated by cigarette smoke (Mian et al., 2008). Treatment of neutrophils with cigarette smoke extract can suppress neutrophil caspase-3-like activity and the phagocytic uptake

and intracellular digestion of staphylococci by neutrophils (Stringer et al., 2007).

### *IL-2 and IL-15*

More than 30 years ago, IL-2 was shown to be a T cell growth factor purified from mitogen-stimulated lymphocyte culture (Morgan et al., 1976). Twenty years later, IL-15 was also identified through its ability to mimic IL-2-induced T cell proliferation (Bamford et al., 1996, Grabstein et al., 1994). Both cytokines belong to the 4-$\alpha$-helix bundle cytokine family and they share several signaling pathways through their heterotrimeric receptors (Lin and Leonard, 1997, Lin et al., 1995, Miyazaki et al., 1994, Fujii et al., 1995, Kawahara et al., 1995, Waldmann and Tagaya, 1999). Receptors for IL-2 (IL-2R) and IL-15 (IL-15R) are comprised of three subunits, namely, IL-2R$\alpha$ (IL-15R$\alpha$ for IL-15), IL-2/15R$\beta$ (also known as CD122) and common cytokine-receptor $\gamma$-chain ($\gamma$c) (Anderson et al., 1995, Asao et al., 2001, Cosman et al., 1995). To achieve high affinity form of receptor, three subunits must be presented together. In the absence of IL-2R$\alpha$ or IL-15R$\alpha$, receptors for IL-2 or IL-15 will only show intermediate-affinity. Signal inductions are relied on $\beta$ and $\gamma$ chains of receptors (Minami et al., 1993, Smith, 1988a, Smith, 1988b, Waldmann et al., 1998). IL-2R$\alpha$ and IL-15R$\alpha$ are two unique subunits for

IL-2 and IL-15 respectively with also distinct physiologic expression profiles. IL-2Rα binds to IL-2 with low-affinity without signal transduction, they are only detectable on activated lymphocytes and Treg cells (Fontenot et al., 2005, Rogers et al., 1997). Whereas IL-15Rα binds to IL-15 with high-affinity and recent studies have shown that IL-15Rα plays functions on intracellular signals through binding to tumour-necrosis factor-receptor-associated factor 2 (TRAF2) through its cytoplasmic tail and preventing interaction between IL-15Rα and the pro-apoptotic cascade members (Bulfone-Paus et al., 1999, Pereno et al., 1999, Pereno et al., 2000). IL-15Rα has a wide cellular distribution. It can be observed in multiple tissues and cell types, such as in liver, heart, spleen, lung and activated vascular endothelial cells (Giri et al., 1995), T cells, B cells, activated monocytes, dendritic cells, macrophages and bone marrow stromal cell lines (Anderson et al., 1995). Distribution of three subunits of IL-2R and IL-15R, namely, α-chain, β-chain (CD122) and γ-chain, is shown in Table 3.2.1.

**Table 3.2.1 Expression of three units of IL-2R and IL-15R on immune cells**

| Cell types | IL-2Rα | IL-15Rα | IL-2/15Rβ | γc |
|---|---|---|---|---|
| Pre-B cells | + | + | - | + |
| Mature B cells | - | + | - | + |
| Mature T cells | | | | |
| Naïve | - | + | + | + |
| Effector | + | + | + | + |
| Memory | - | + | + | + |
| Treg cells | + | - | + | + |
| NK cells | - | + | + | + |
| Dendritic cells | +/- * | + | +/-* | + |
| Monocytes | - | + | - | + |

*Certain activated dendritic cells have been shown to express IL-2Rα and IL-2/15Rβ on their surfaces (Ardavin and Shortman, 1992, Mnasria et al., 2008).

Due to common features in receptor components and signaling pathways, IL-2 and IL-15 have several similar functions. They can induce the proliferation of activated T cells, generate cytotoxic T cells and NK cells and facilitate B cell proliferation and immunoglobulin synthesis (Fehniger and Caligiuri, 2001, Waldmann et al., 2001, Waldmann and Tagaya, 1999). However, in many adaptive immune responses, significant differences between IL-2 and IL-15 have been identified in their molecular and cellular biology and functions (Thomas, 2008, Waldmann and Tagaya, 1999). The distinct binding affinity and distribution of two α-chains may be one reason contributing to distinct features of two receptors. As mentioned previously, IL-2α is mainly expressed by activated

T cells and bind IL-2 with low affinity, while IL-15$\alpha$ binding to IL-2 with high affinity is widely spread in many tissues and cell types. Also, in contrast to IL-2 that binds pre-formed high affinity heterotrimeric receptors at the cell surface (Taniguchi and Minami, 1993, Waldmann, 1991), IL-15 is only secreted at low levels and binds membrane IL-15R (Dubois et al., 2002). Functional differences may result in distinctive roles of IL-2 and IL-15 in human diseases. For example, IL-2 can down-regulate $\gamma$ chain expression on cycling T cells and dampen T cell clonal expansion through activation-induced cell death (AICD) (Lenardo, 1991), whereas IL-15 is an anti-apoptosis factor and inhibits IL-2 induced AICD by facilitating the maintenance of $CD8^+$ T cell survival, including self-reactive memory T cells (Marks-Konczalik et al., 2000). Furthermore, IL-2 participates in the maintenance of peripheral Treg cells (D'Cruz and Klein, 2005, Fontenot et al., 2005, Maloy and Powrie, 2005) but IL-15 has no marked effects on them (Fehniger and Caligiuri, 2001a). IL-2 shows ability of inhibiting $CD8^+$ memory T cell proliferation but IL-15 can selectively stimulated the proliferation of memory $CD8^+$ T cells and prolong their survival (Zhang et al., 1998). Collectively, although both IL-2 and IL-15 play potent roles in the pathogenesis of autoimmune diseases, cancers and inflammatory diseases, they can be either beneficial of

**Table 3.2.2 Comparison of human IL-2 and IL-15**

| Properties | IL-2 | IL-15 |
|---|---|---|
| **Structure** | 15.5kDa; four-helix bundle | 14-15kDa; four-helix bundle |
| **Receptors** | IL-2Rα, low affinity; IL-2/15Rβ; γc | IL-15Rα, high affinity; IL-2/15Rβ; γc |
| **Producing cells** | Activated T cells | Monocytes, dendritic cells and epithelial cells* |
| **Function** | Proliferation of T cells, NKT cells, NK cells and B cells;<br>Activating NK cells and NKT cells;<br>Elimination of self-reactive T cells;<br>Inducing AICD;<br>Maintenance of Treg Cells | Proliferation and activation of NKT cells and NK cells;<br>Maintenance of memory CD8+ T cells and NK cell survival;<br>Inhibiting IL-2-induced AICD |

* Epithelial cells are big producers of IL-15. Respiratory viruses such as respiratory syncytial virus (RSV) increased surface MIC molecules in respiratory epithelial cells (Zdrenghea et al., 2012). These molecules are ligands for NKG2D receptors on NK and T cells, playing an important role in the nonspecific activation of cytotoxic activity of NKG2D-positive NK and $CD8^+$ T cells.

detrimental. Their application in immune therapy may also differ at some points due to their unique biological features. Table 3.2.2 simply outlines the comparison of human IL-2 and IL-15.

To date, IL-2 and IL-15 biological functions and their therapeutic applications have been well studied in cancers, viral infections and certain autoimmune diseases.

Nevertheless, their properties in smokers or COPD patients are still less explored. Higher IL-2 levels have been found in lung plasma samples from stable COPD patients compared to participants with rapidly progressive COPD (D'Armiento et al., 2009). Also, corticosteroid (dexamethasone) have shown less inhibiton of IL-2 production in COPD patients compared to healthy volunteers although these numerical differences between groups did not reach statistical significance (Kaur et al., 2012). *In vitro*, it has shown that IL-15 stimulation by itself had no effect on IFN-$\gamma$ protein expression in COPD patients at GOLD stages 2, 3, and 4. However, IL-15 primed $CD8^+$ lung T cells to have greater cytotoxic potential by expressing higher levels of intracellular perforin when they encounter a target cells in COPD (Freeman et al., 2010). Furthermore, CSE treated NK cells were less activated after poly I:C stimulation, and this suppression was IL-15 mediated (Mian et al., 2009).

Accumulating evidence strongly suggest CSE has markedly association with COPD developments. Although numbers of studies have shown that CSE impairs cytokine and chemokine productions in many cell types, for instance, macrophages, neutrophils, DCs or other inflammatory cells in healthy volunteers (Gaschler et al., 2008, Takahashi et al., 2007,

Takahashi et al., 2006), however, the impact of CSE on killer cells from COPD patients remains to be explored. Previous section has shown that killer cells were all significantly activated *in vivo* in current smokers with or without COPD, which indicates that cigarette smoking, rather than disease itself, may play a potent role in killer cell activation in peripheral blood. $\alpha$-CD3 is a great stimulator for $CD3^+$ T cell activation. Whereas, IL-2 and IL-15 can induce NKT cells and NK cell stimulation. In this chapter, we have investigated, *in vitro,* the effects of CSE on unstimulated or $\alpha$-CD3, IL-2 or IL-15 activated killer cells including $CD8^+$ T cells, NKT-like cells and NK cells, from peripheral blood in four designed groups, namely, healthy non-smokers (HNS), current healthy smokers (HS), current smokers with COPD (cuS-COPD) and ex-smokers with COPD (exS-COPD).

### 3.2.2 Material and methods

#### 3.2.2.1 Study population and procedures

The criteria of participant enrollments are the same as previously mentioned. Twenty participants were included in CSE activation study. Table 3.2.3 and Table 3.2.4 detail the demographic and spirometric data of the participants in

**Table 3.2.3 Demographic and spirometric valus of the healthy non-smoking participants in optimal concentration experiments**

| Healthy non-smokers | Age (years) | Gender (M/F) | FEV1 (% pred) | $FEV_1$/ FVC (%) | $\Delta FEV_1$ post bronch | BMI ($kg/m^2$) |
|---|---|---|---|---|---|---|
| **n=4** | 49 (43-57) | 3/1 | 110 (89-118) | 99 (82-111) | 2.3 (2.0-2.8) | 22.8 (19.6-27.7) |

**Table 3.2.4 Demographic and spirometric valus of the CSE suppression study groups**

| | Healthy non-smokers | Healthy smokers | cuS-COPD | exS-COPD |
|---|---|---|---|---|
| **Participants** | 4 | 4 | 4 | 4 |
| **Age (years)** | 55 (53-60) | 54 (50-58) | 64 (55-73) | 56 (49-65) |
| **Gender (M/F)** | 1/3 | 3/1 | 3/1 | 2/2 |
| **Packs/yrs** | 0 | 36 (32-40) | 47 (42-66) | 34 (23-47) |
| **Smoking status (Current/Ex)** | 0 | 4/0 | 4/0 | 0/4 |
| **Chronic bronchitis (Yes/No)** | 0 | 2/2 | 3/1 | 1/3 |
| **$FEV_1$ (% pred)** | 103 (81-116) | 98 (89-106) | 43 (37-52) | 59 (54-63) |
| **$FEV_1$/FVC (%)** | 94 (76-108) | 83 (70-99) | 36 (32-46) | 52 (37-57) |
| **$\Delta FEV_1$ post bronch** | 2.3 (2.0-2.6) | 2.0 (1.9-2.4) | 1.6 (1.5-2.2) | 1.2 (0.9-1.3) |
| **BMI ($kg/m^2$)** | 23.6 (20.7-26.0) | 26.0 (21.8-28.5) | 25.9 (18.9-29.8) | 21.1 (20.0-22.7) |
| **Inhaled GCS (on/off)** | N/A | N/A | 1/3 | 1/3 |
| **MRC dyspnoea scale** | N/A | N/A | 3 (2-4) | 3 (2-4) |
| **Distance walked in 6 min (m)** | N/A | N/A | 268 (102-364) | 336 (152-428) |
| **BODE Index** | N/A | N/A | 5 (3-6) | 4 (2-5) |

preliminary concentration experiments and CSE suppression study respectively.

**Table 3.2.5 Antibodies used for flow cytometry**

| Antigen | Fluorochrome | Isotype | Clone | Source |
|---|---|---|---|---|
| CD3 | PC7 | Mouse IgG1 | UCHT1 | Beckman Coulter, Luton, UK |
| CD8 | ECD | Mouse IgG1 | SFCI21Thy2D3 | Beckman Coulter, Luton, UK |
| CD25 | FITC | Mouse IgG2a, | B1.49.9 | Beckman Coulter, Luton, UK |
| CD56 | PE | Mouse IgG1 | N901 | Beckman Coulter, Luton, UK |
| CD69 | PC5 | Mouse IgG2b | TP1.55.3 | Beckman Coulter, Luton, UK |

### 3.2.2.2 Reagents

Anti-CD3 was purchased from R&D system (Abingdon, UK). Recombined human IL-2 (rhIL-2) and recombined human IL-15 (rhIL-15) were obtained from PeproTech EC (London, UK). Other monoclonal antibodies for flowcytometry were shown in table 3.2.5.

### 3.2.2.3 Peripheral Blood Mononuclear Cell isolation

PPBMCs were isolated from whole blood on a discontinuous histopaque density gradient (Sigma, Poole, UK). More details are provided in section 2.2.

### 3.2.2.4 Cigarette smoke extract preparation

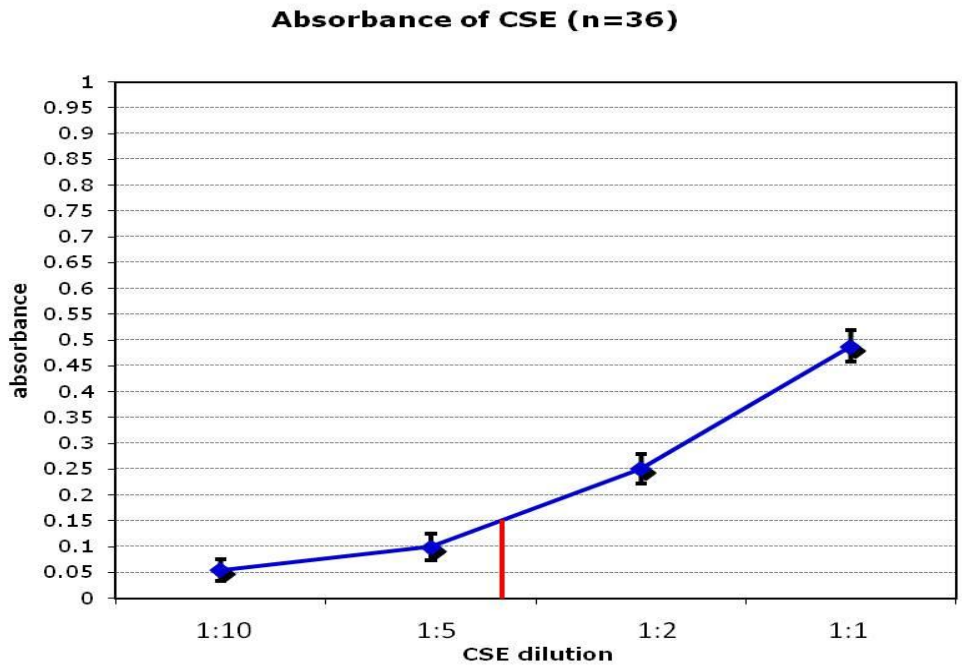



**Figure 3.2.1 Absorbance of CSE at different dilutions.**

The absorbance of 0.15 is considered as 100% CSE.

CSE were freshly prepared prior to 30 minutes of cell culture for each experiment to avoid breakdown of substances in the extract and evaporation of volatile components. More details are provided in section 2.4. Figure 3.2.1 shows the trend of absobance according to CSE dilution. Thirty-six (n=36) cigarette were processed in this manner during the whole project.

### 3.2.2.5 PBMCs culture and treatments

In preliminary experiment to determine the optimal culture time and concentrations of CSE, α-CD3, IL-2 or IL-15 for

**Table 3.2.6 Concentrations of stimulus used for activating killer cell.**

| CSE | 0.2% | 0.5% | 1% | 2% | 5% |
|---|---|---|---|---|---|
| α-CD3 | 0.1ug/ml | 0.2ug/ml | 0.5ug/ml | 1ug/ml | 2ug/ml |
| IL-2 | 5ng/ml | 10ng/ml | 20ng/ml | 50ng/ml | 100ng/ml |
| IL-15 | 1ng/ml | 2ng/ml | 5ng/ml | 10ng/ml | 20ng/ml |

activation of killer cells, PBMCs isolated from healthy non-smoking participants were resuspended in RPMI-1640 medium supplemented with 10% FCS at a density of $1x10^6$ cells/ml and cultured at 200ul/well in 96-well flat bottom tissue culture plates. These cells were either untreated or treated separately with CSE,  -CD3, IL-2 or IL-15 at various concentrations as indicated in Table 3.2.6. Specially, α-CD3 was pre-coated in the plate for 90 minutes at 37°C in 5% $CO_2$ and washed in cold sterile D-PBS twice to remove the unbound antibody. After incubation for 24 hours, 48 hours and 72 hours, cells were harvested at each time point and washed twice in RPMI-1640 medium for surface marker staining to detect cell activation.

In CSE suppression experiments, PBMCs isolated from HNS, HS, cuS-COPD and exS-COPD were untreated or treated with CSE, α-CD3, IL-2 or IL-15 at each optimized concentrations seperately. In order to test the effects of CSE on activated

killer cells, cells stimulated by $\alpha$-CD3, IL-2 or IL-15 were also co-treated by CSE. For CSE suppression experiment, cells were incubated for 48 hours at 37°C in 5% $CO_2$ before analysis.

### 3.2.2.6 Extracellular staining

Fluorescently conjugated antibodies used in this study for extracellular staining were as follows, CD3 (PC7), CD56 (PE), CD8 (ECD) CD25 (FITC) and CD69 (PC5), or KIR (FITC). More details are provided in section 2.6.

### 3.2.2.7 Flow cytometric analysis

Untreated or treated PBMCs were analysed by flow cytometry. Cells were harvested and fixed in 3% formaldehyde in isotonic azide free solution (Beckman Coulter, Luton, UK) and washed before labelled antibodies were added at the recommended concentration. The cells were incubated in the dark and excess antibody removed. Flow cytometric analysis of antibody labelled cells was performed using an EPICS Altra (Beckman Coulter, Luton, UK). More details are provided in section 2.9.

### 3.2.2.8 Statistical analysis

The statistical analysis was performed with Prism software, version 4.0c (GraphPad). Differences between the four groups

of participants were tested using the parametric student's *t* test. Confidence interval 99% was used to show two-tailed p values. P values of less than 0.01 were considered to indicate statistical significance due to the small size of each study group..

### 3.2.3 Results

#### 3.2.3.1 Optimal concentrations and culture time for killer cell activation

To determine the optimal concentrations of interested stimulus and culture time, PBMCs from healthy non-smokers (n=4) were cultured in medium alone or in medium supplemented with either CSE, α-CD3, IL-2 or IL-15 at increasing concentrations (as shown in Table 3.2.6) for 24 hours, 48 hours and 72 hours. Cells are designated activated when they express one or both of the activation markers (i.e., CD69 positive, CD69/CD25 double positive or CD25 positive).

Dose- and time-dependent increases in percentages of killer cell activation were observed during culture with increasing concentrations of α-CD3 (Figure 3.2.2), IL-2 (Figure 3.2.3) and IL-15 (Figure 3.2.4).

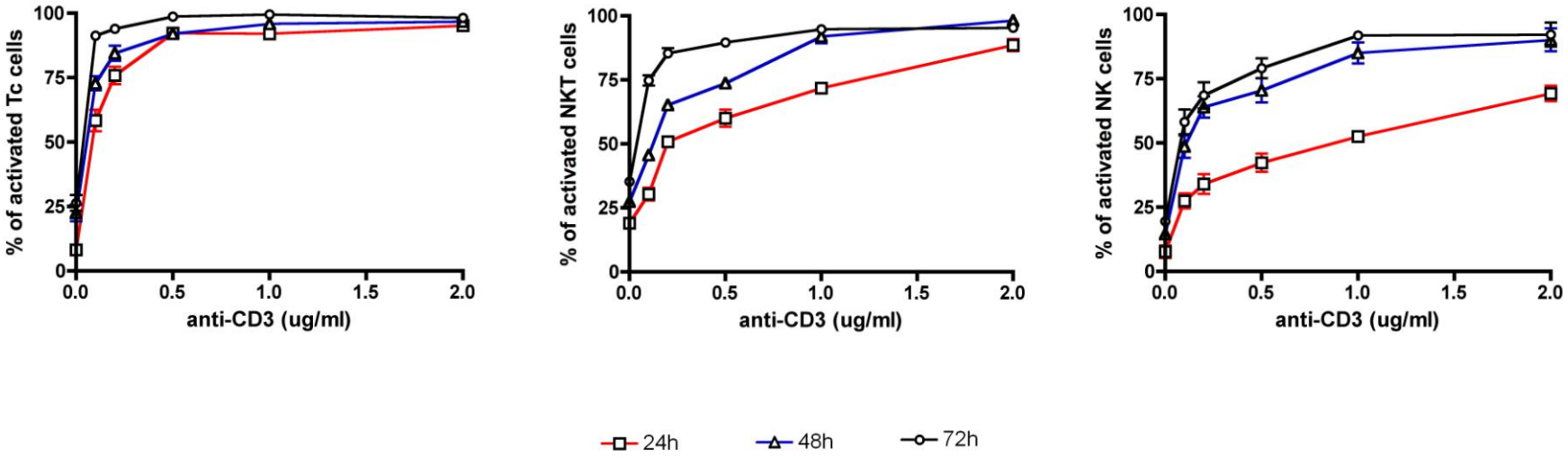



**Figure 3.2.2 Activation levels of killer cells by different concentrations of α-CD3.** PBMCs were collected from healthy non-smokers (n=4). Cells were untreated or treated by α-CD3 at concentrations of 0.1ug/ml, 0.2ug/ml, 0.5ug/ml, 1ug/ml and 2ug/ml respectively for 24 hours, 48 hours and 72 hours.

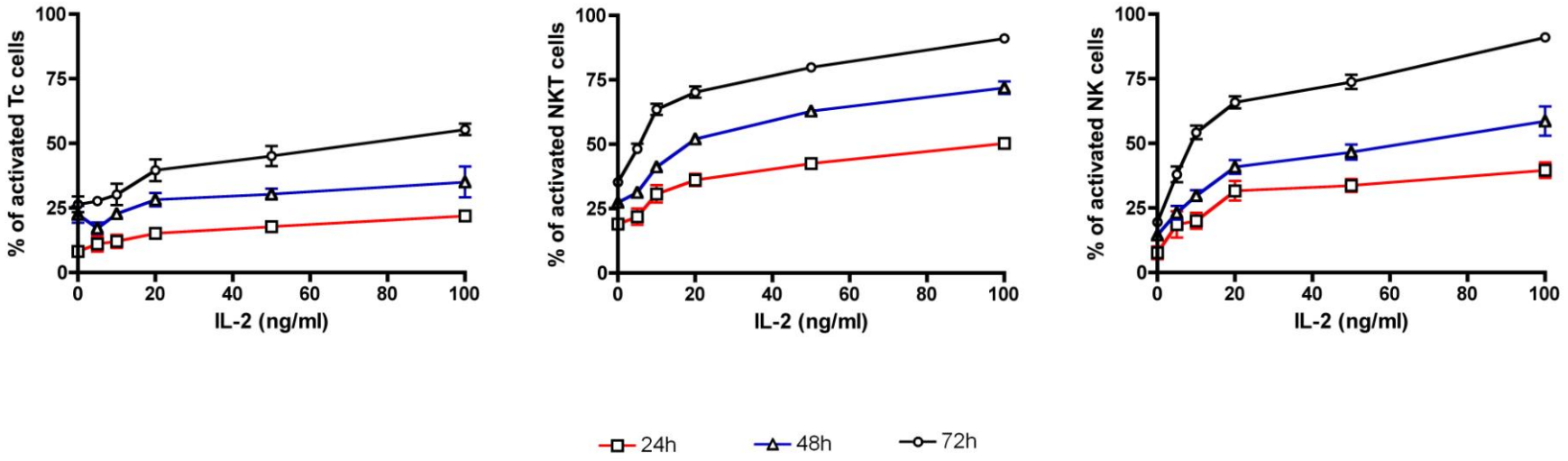



**Figure 3.2.3 Activation levels of killer cells by different concentrations of IL-2.** PBMCs were collected from healthy non-smokers (n=4). Cells were untreated or treated by IL-2 at concentrations of 5ng/ml, 10ng/ml, 20ng/ml, 50ng/ml and 100ng/ml respectively for 24 hours, 48 hours and 72 hours.

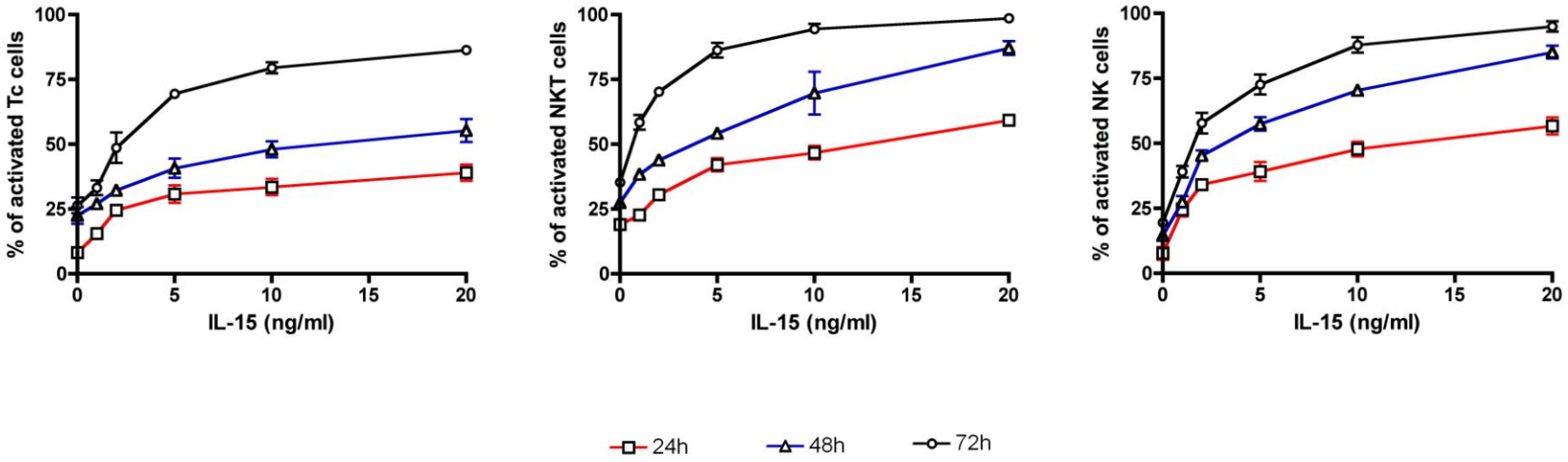



**Figure 3.2.4 Activation levels of killer cells by different concentrations of IL-15.** PBMCs were collected from healthy non-smokers (n=4). Cells were untreated or treated by IL-15 at concentrations of 1ng/ml, 2ng/ml, 5ng/ml, 10ng/ml and 20ng/ml respectively for 24 hours, 48 hours and 72 hours.

Killer cells of interest including Tc cells, NKT-like cells and NK cells were highly activated when increasing the concentration of $\alpha$-CD3 to 1ug/ml after incubation for 48 hours (Figure 3.2.2). Activations of killer cells by IL-2 at selected concentrations were not as much activation as $\alpha$-CD3 treated cells (Figure 3.2.3). However, previous studies have shown that administration of high dose of IL-2 in treatment of a variety of diseases is associated with mortality rates of up to 4% . Therefore, we controlled the maximal concentration of IL-2 at 100ng/ml. IL-15 at 20ng/ml also highly activated these killer cells after 48 hours incubation (Figure 3.2.4).

There were no statistically significant differences on the activation levels of killer cells treated by CSE at different percentage concentrations (all $p>0.05$) (Figure 3.2.5).

To sum up, to highly activate all killer cells of interest, the optimal concentration of $\alpha$-CD3, IL-2, IL-15 for detection of cell activation would be chosen at 1ug/ml, 100ng/ml, and 20ng/ml respectively in future experiments. The dilution at 1% of CSE would also be used for following studies. Figure 3.2.6 showed representative dot plots of interested killer cell activation at optimal concentration and dilution after 48 hours incubation.

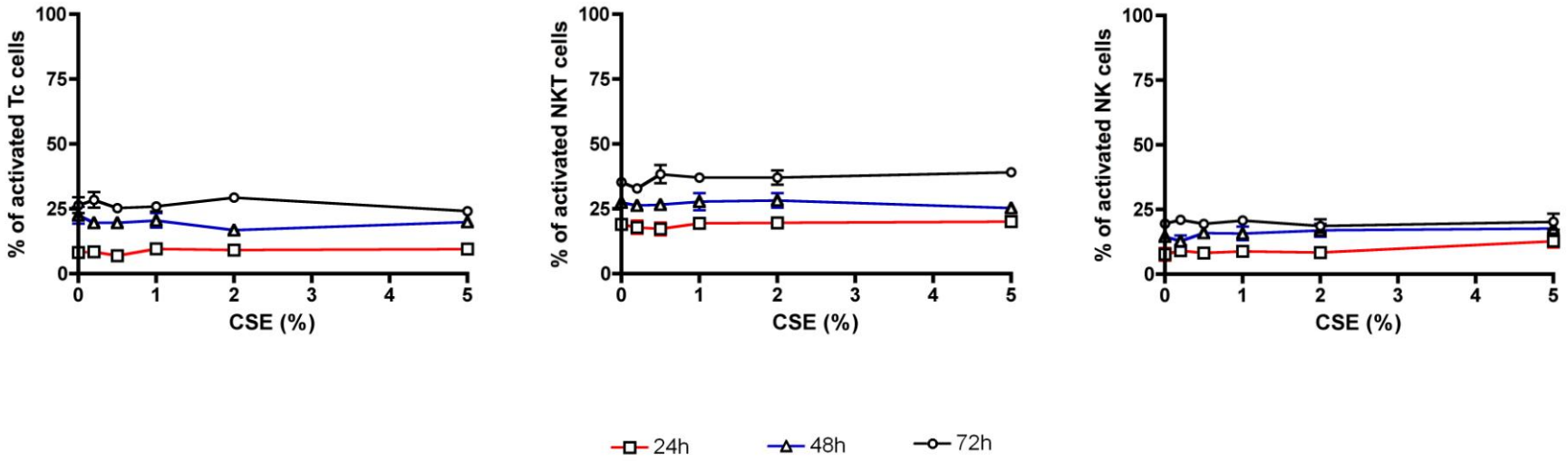



**Figure 3.2.5 Activation levels of killer cells by different dilutions of CSE.** PBMCs were collected from healthy non-smokers (n=4). Cells were untreated or treated by CSE at dilutions of 0.2%, 0.5%, 1%, 2% and 5% respectively for 24 hours, 48 hours and 72 hours.

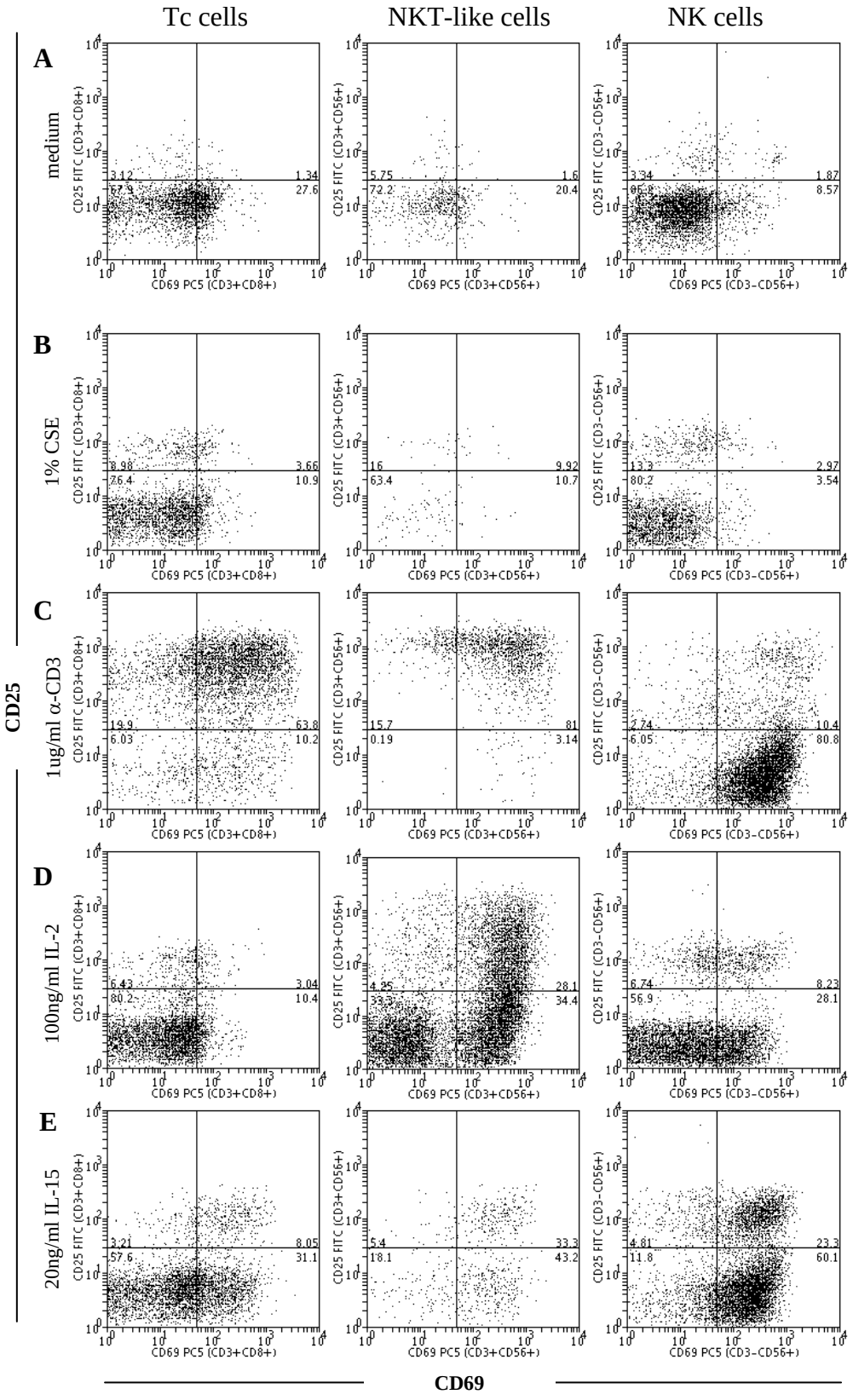
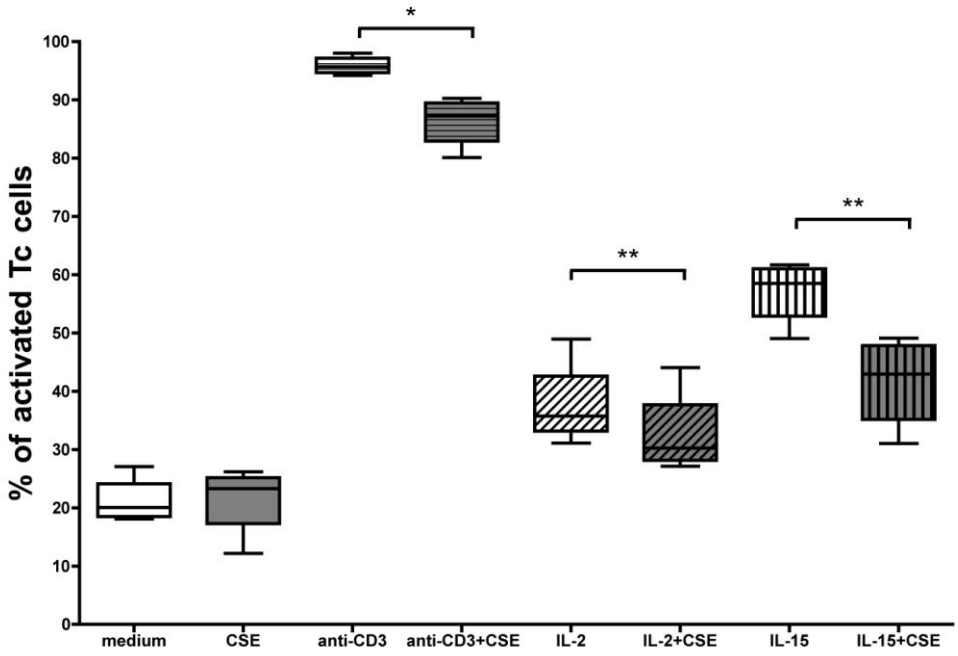



**Figure 3.2.6 Representative dot plots for activation of Tc cells, NKT-like cells and NK cells in PBMCs from healthy non-smokers (n=4) by different stimulus at 48 hours.** A) medium only; B) 1% CSE; C) 1ug/ml α-CD3; D) 100ng/ml IL-2; and E) 20ng/ml IL-15.

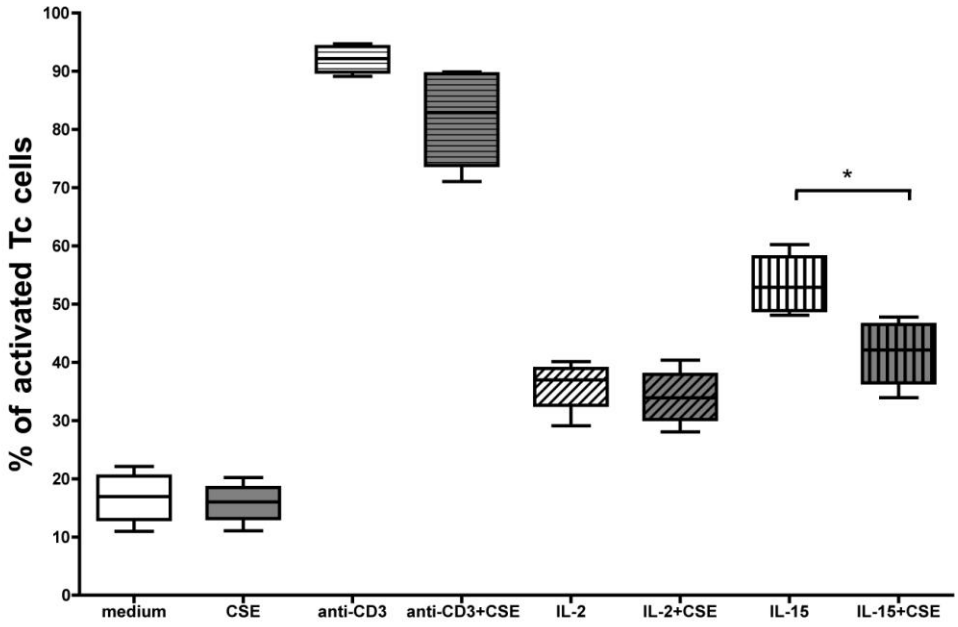
### 3.2.3.2 CSE suppressed killer cell activation in response to different stimulus

PBMCs collected from the four study groups were either untreated (control) or treated by 1% of CSE alone, or incubated with 1ug/ml $\alpha$-CD3, 100ng/ml IL-2 or 20ng/ml IL-15 for 48 hours in the absence or presence of 1% of CSE. There was no significant difference on the activation of $CD8^+$ T cells (Tc cells) between untreated PBMCs and CSE treated PBMCS in each group (Figure 3.2.7). CSE significantly reduced the activation levels of anti-CD3 treated Tc cells from healthy non-smokers (Figure 3.2.7.A, $p<0.01$) and current smokers with COPD (Figure 3.2.7.C, $p<0.005$). Also, IL-2 indcued Tc cell activation was dramatically decreased when CSE was added in healthy non-smokers (Figure 3.2.7.A, $p<0.005$), current smokers with COPD (Figure 3.2.7.C, $p<0.001$) and ex-smokers with COPD (Figure 3.2.7.D, $p<0.001$). Furthermore, CSE markedly attenuated IL-15 stimulated T cell activation in all interested groups (p value was shown in Table 3.2.7).

**A. Healthy non-smokers (HNS)**



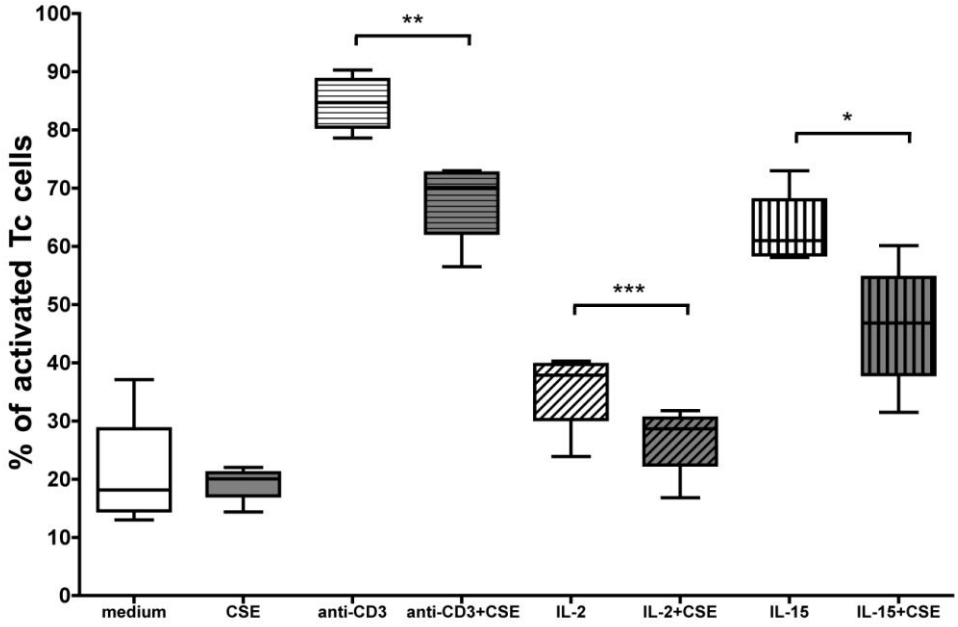

**B. Current Healthy smokers (HS)**




CSE (1%); anti-CD3 (1ug/ml)

IL-2 (100ng/ml); IL-15 (20ng/ml)

**C. current smokers with COPD (cuS-COPD)**




**D. ex-smokers with COPD (exS-COPD)**

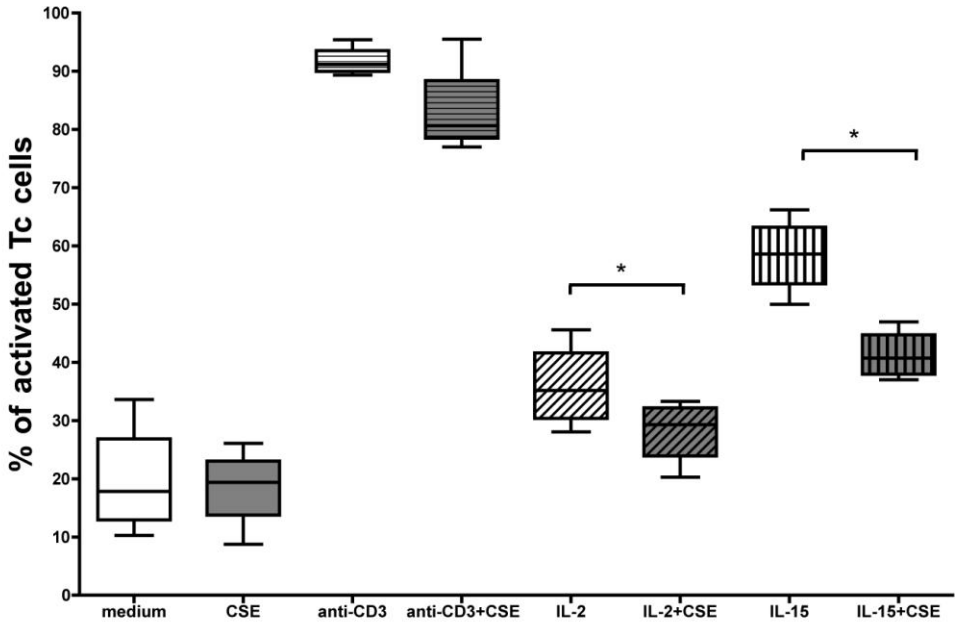



**Figure 3.2.7 CSE effects on activation levels of Tc cells in four groups.** PMBCs were collected from four groups including A) HNS (n=4), B) HS (n=4), C) cuS-COPD (n=4), and D) exS-COPD (n=4). Cells were untreated or treated by different reagents at designed concentrations and incubated 48 hours. *, $p<0.01$; **, $p<0.005$; ***, $p<0.001$.

**Table 3.2.7 Statistical difference of before and after CSE treated cells**

| | | Medium *vs* CSE | anti-CD3 *vs* anti-CD3+CSE | IL-2 *vs* IL-2+CSE | IL-15 *vs* IL-15+CSE |
|---|---|---|---|---|---|
| **Tc cells** | **HNS** | p>0.01 | **p<0.01** | **p<0.005** | **p<0.005** |
| | **HS** | p>0.01 | p>0.01 | p>0.01 | **p<0.01** |
| | **cuS-COPD** | p>0.01 | **p<0.005** | **p<0.001** | **p<0.01** |
| | **exS-COPD** | p>0.01 | p>0.01 | **p<0.01** | **p<0.01** |
| **NKT-like cells** | **HNS** | p>0.01 | **p<0.005** | **p<0.01** | **p<0.01** |
| | **HS** | p>0.01 | p>0.01 | p>0.01 | **p<0.005** |
| | **cuS-COPD** | p>0.01 | **p<0.005** | **p<0.005** | **p<0.01** |
| | **exS-COPD** | p>0.01 | **p<0.005** | **p<0.01** | **p<0.005** |
| **NK cells** | **HNS** | p>0.01 | **p<0.001** | **p<0.01** | **p<0.005** |
| | **HS** | p>0.01 | **p<0.005** | **p<0.005** | **p<0.001** |
| | **cuS-COPD** | p>0.01 | **p<0.005** | **p<0.005** | **p<0.001** |
| | **exS-COPD** | p>0.01 | **p<0.005** | **p<0.005** | **p<0.001** |

- HNS, healthy non-smokers; HS, current healthy smokers; cuS-COPD, current smokers with COPD; exS-COPD, ex-smokers with COPD.
- CSE showed significant effects on all cell types of interest upon IL-15 stimulation in all groups.

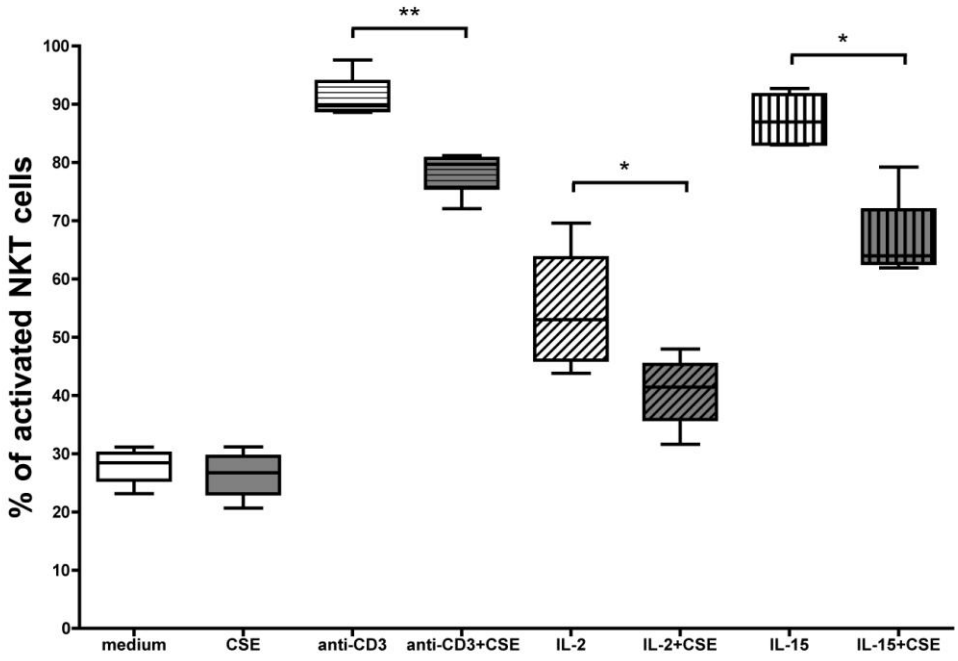
CSE effects on activated NKT cells were also investigated. There was no significant difference on the activation levels of NKT-like cells treated with or without CSE in each group (Figure 3.2.8). CSE significantly reduced the activation levels of $\alpha$-CD3 treated Tc cells from healthy non-smokers (Figure 3.2.8.A, $p<0.005$), current smokers with COPD (Figure 3.2.8.C, $p<0.005$) and ex-smokers with COPD (Figure 3.2.8.D, $p<0.005$). IL-2 indcued NKT-like cell activation was dramatically decreased when CSE was added in healthy non-smokers (Figure 3.2.8.A, $p<0.01$), current smokers with COPD

(Figure 3.2.8.C, $p<0.005$) and ex-smokers with COPD (Figure 3.2.8.D, $p<0.01$). Furthermore, CSE markedly attenuated IL-15 stimulated NKT-like cell activation in all groups (p value was shown in Table 3.2.7).

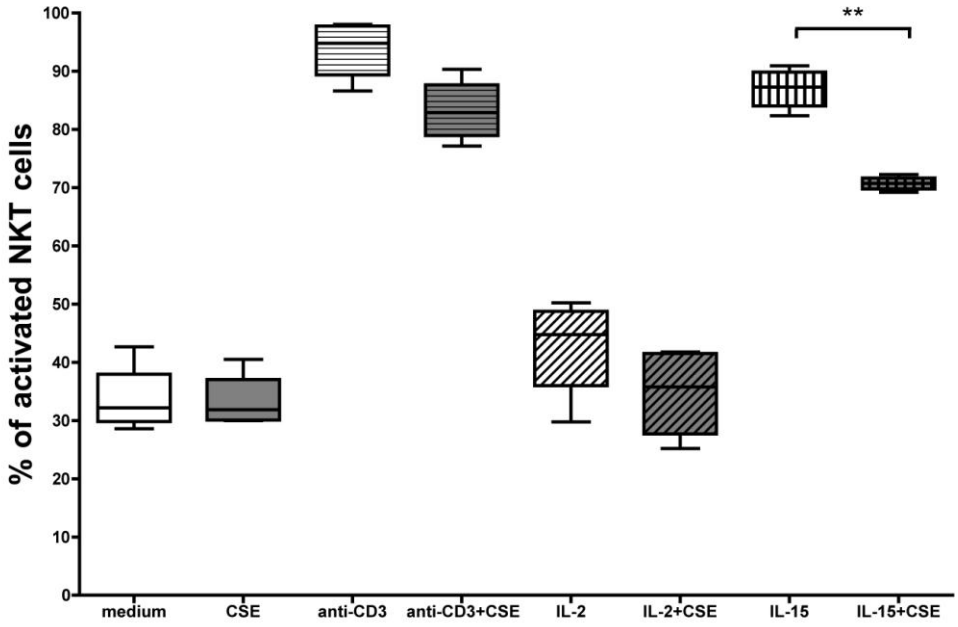
When examining activated levels on NK cells, untreated cells compared to CSE alone had no markedly effects on NK cell activation in each patient group (Figure 3.2.9). Activation levels of NK cell in response to $\alpha$-CD3, IL-2 or IL-15 were all significangly decreased in the presence of CSE (p value was shown in Table 3.2.7).

Absolute acitvation levels of killer cells in response to each stimulation were also compared between groups. There were no statistical differences detected (Figures are shown in Appendix).

**A. Healthy non-smokers (HNS)**



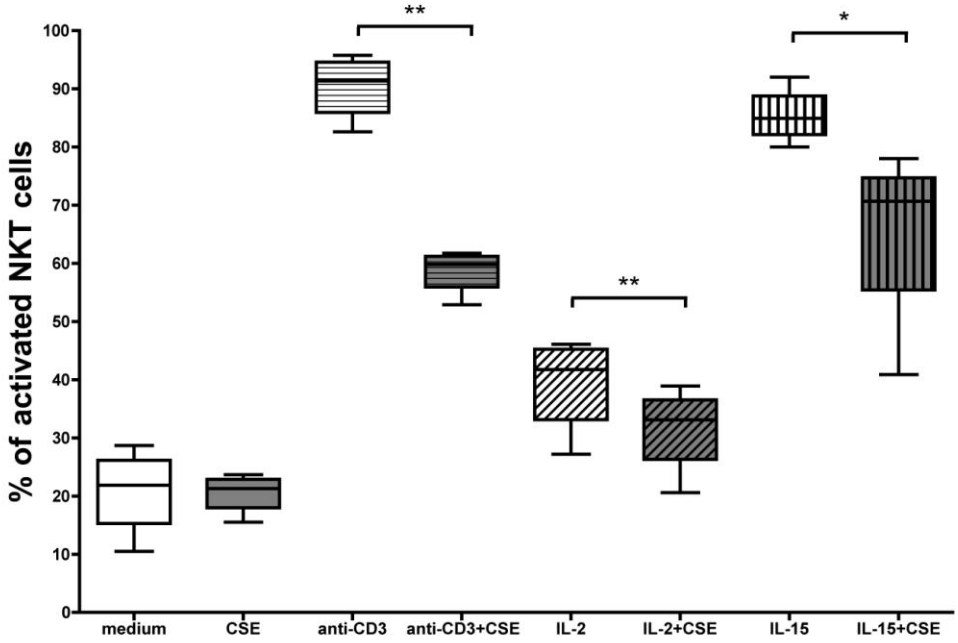

**B. Current healthy smokers (HS)**




CSE (1%); anti-CD3 (1ug/ml)

IL-2 (100ng/ml); IL-15 (20ng/ml)

**C. current smokers with COPD (cuS-COPD)**




**D. ex-smokers with COPD (exS-COPD)**

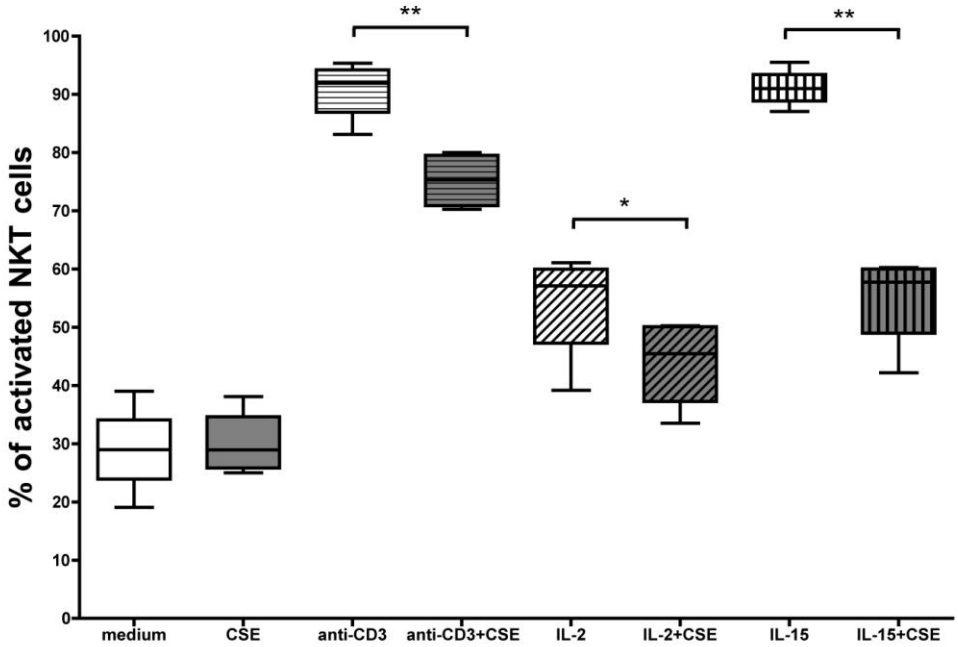
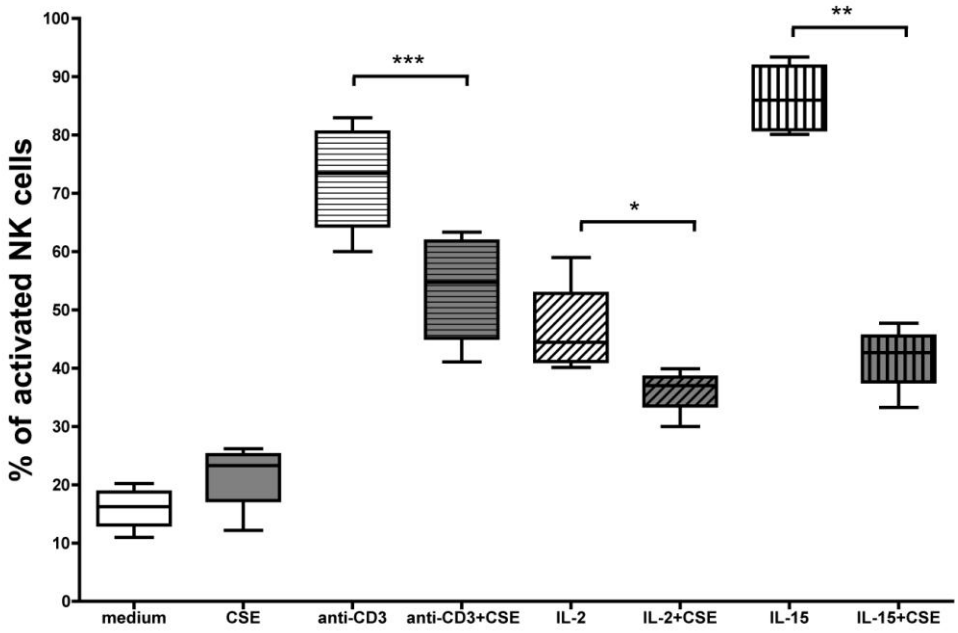
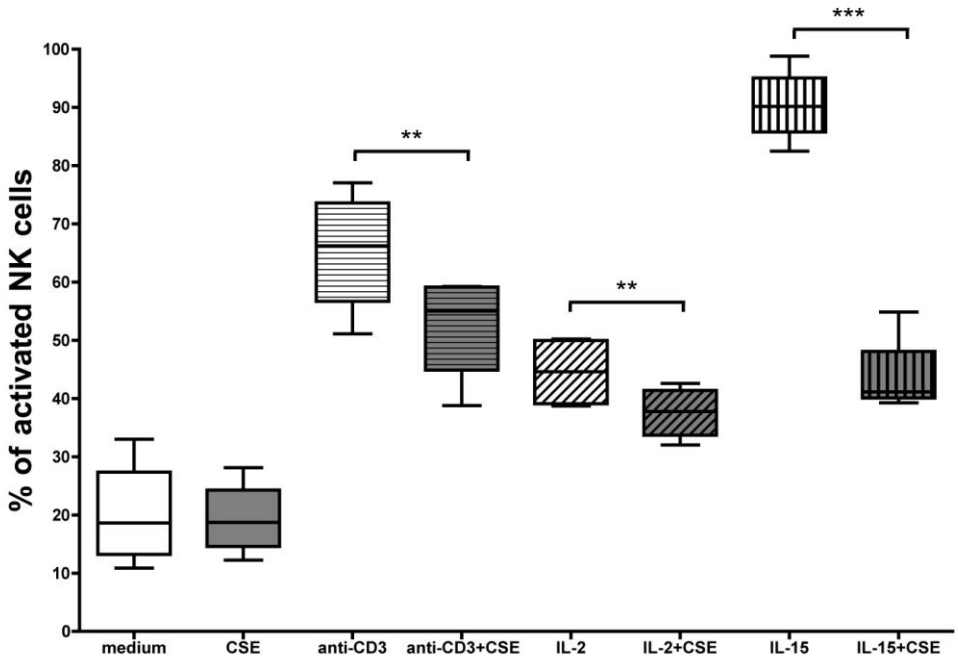



**Figure 3.2.8 CSE effects on activation levels of NKT cells in four groups.** PMBCs were collected from four groups including A) HNS (n=4), B) HS (n=4), C) cuS-COPD (n=4), and D) exS-COPD (n=4). Cells were untreated or treated by different reagents at designed concentrations and incubated 48 hours. *, $p<0.01$; **, $p<0.005$; ***, $p<0.001$.

**A. Healthy non-smokers (HNS)**



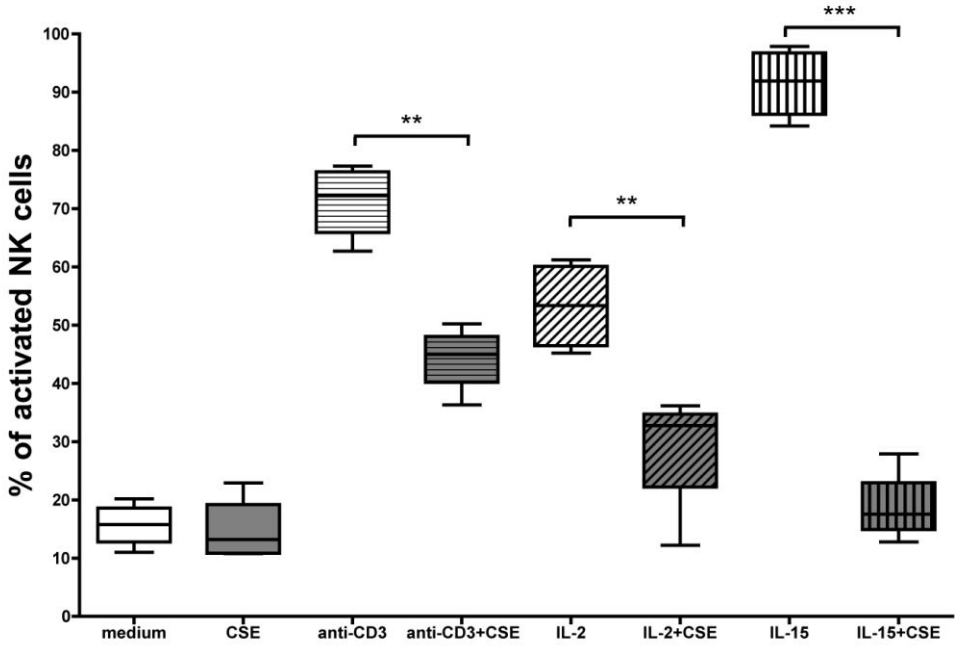

**B. Current healthy smokers (HS)**




CSE (1%); anti-CD3 (1ug/ml)

IL-2 (100ng/ml); IL-15 (20ng/ml)

**C. current smokers with COPD (cuS-COPD)**




**D. ex-smokers with COPD (exS-COPD)**

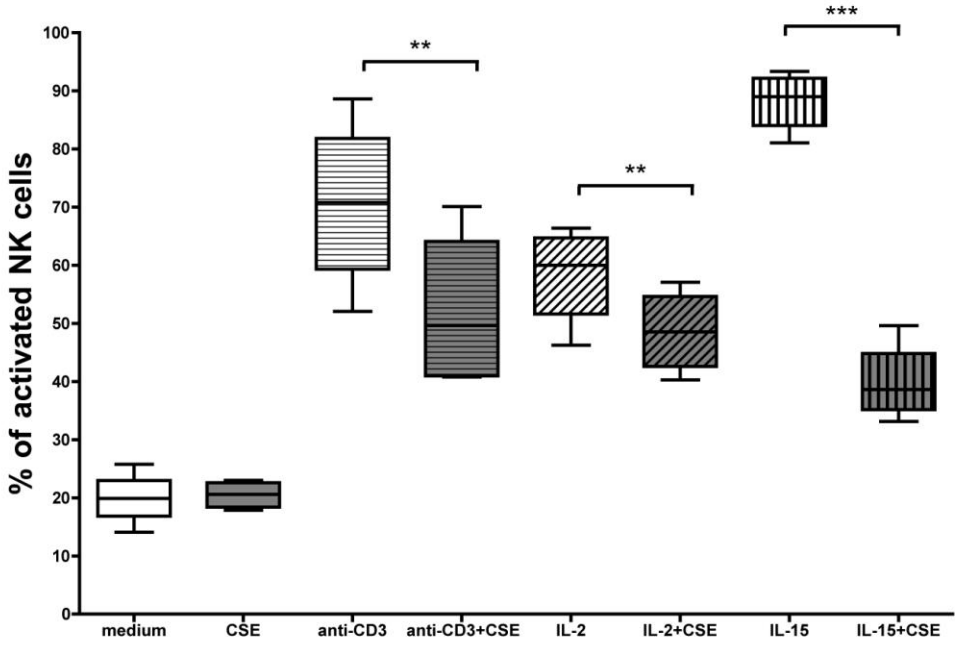



**Figure 3.2.9 CSE effects on activation levels of NK cells in four groups.** PMBCs were collected from four groups including A) HNS (n=4), B) HS (n=4), C) cuS-COPD (n=4), and D) exS-COPD (n=4). Cells were untreated or treated by different reagent at designed concentrations and incubated 48 hours. *, P<0.01; **, p<0.005; ***, p<0.001.

### 3.2.3.3 Absolute suppression levels induced by CSE on killer cell activation in response to different stimulation

Since CSE dramatically suppressed most activation levels of Tc cells, NKT-like cells and NK cells upon different stimulation in each group, we further compared the absolute suppression levels induced by CSE on cell activation between four groups. The calculation fomula of absolute suppression levels was "Absolute suppression level = the percentage of activated cells of interest up on different stimulation - the percentage of these cells upon CSE treatment". There were no significant differences on the absolute levels of CSE induced suppression in $\alpha$-CD3 treated Tc cell activatioin between four groups (Figure 3.2.10.A, all $p>0.01$). Absolute suppression level of CSE treated NKT cells upon $\alpha$-CD3 stimulation was markedly higher in current smokers with COPD compared to healthy non-smokrers ($p<0.005$), current healthy smokers ($p<0.01$) and ex-smokers with COPD ($p<0.005$) (Figure 3.2.10.B). Furthermore, CSE inducing absolute suppression level on NK cell activation upon $\alpha$-CD3 stimulation was markedly higher in current smokers with COPD compared to healthy non-smokers ($p<0.01$) and current healthy smokers ($p<0.005$) (Figure 3.2.10.C). The absolute suppression level on CSE treated NK cells in response to $\alpha$-CD3 was also significantly

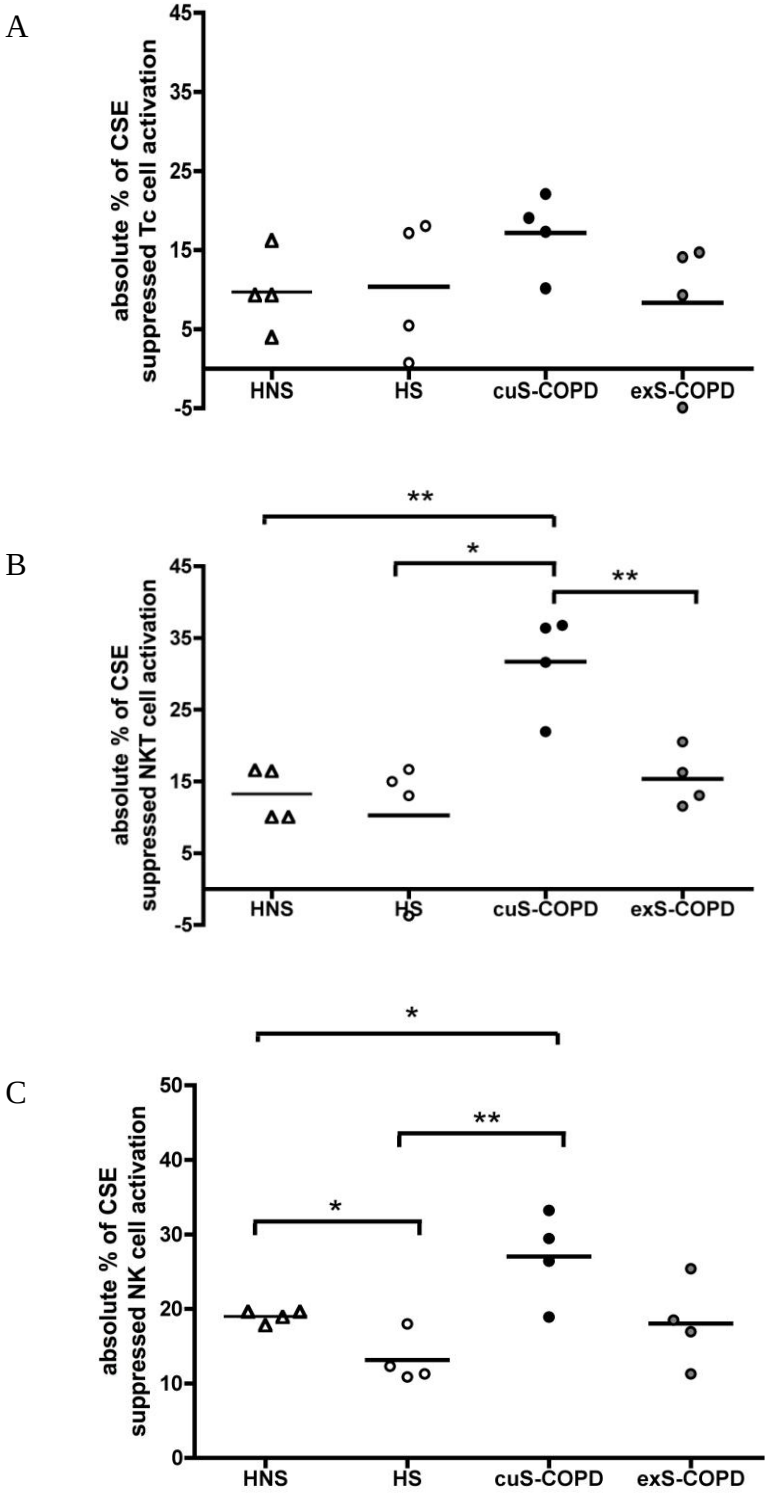



**Figure 3.2.10 Absolute suppression levels induced by CSE on α-CD3 treated killer cell activation in four groups.** A) Tc cells; B) NKT cells; C) NK cells. HNS (healthy non-smokers, n=4); HS (current healthy smokers, n=4); C) cuS-COPD (current smokers with COPD, n=4); exS-COPD (ex-smokers with COPD, n=4). *, P<0.01; **, p<0.005; ***, p<0.001.

higher in healthy non-smokers ($p<0.005$) in contrast to current healthy smokers (Figure 3.2.10.C).

In IL-2 treated Tc cells, absolute suppression levels induced by CSE were significantly higher in current smokers with COPD compared to healthy non-smokers ($p<0.005$) and current healthy smokers ($p<0.01$) (Figure 3.2.11.A). Also, absolute suppression level on CSE treated NK cell activation was remarkably higher in current smokers with COPD compared to healthy non-smokrers ($p<0.01$), current healthy smokers ($p<0.005$) and ex-smokers with COPD ($p<0.005$) (Figure 3.2.11.C). There were no significant differences on the absolute levels of CSE inducing suppression on Tc cell activation between four groups (Figure 3.2.11.B, all $p>0.01$).

CSE did not show any statistical differences on the absolute suppression levels of IL-15 stimulted Tc cells between groups (Figure 3.2.12.A). NKT cells in response to IL-15 was dramatically suppressed when CSE was added in ex-smokers with COPD compared to healthy non-smokers ($p<0.01$) and current healthy smokers ($p<0.001$) (Figure 3.2.12.B). Moreover, the absolute suppression level induced by CSE on IL-15 treated NK cells was significantly higher in current smokers with COPD compared to other three gourps including

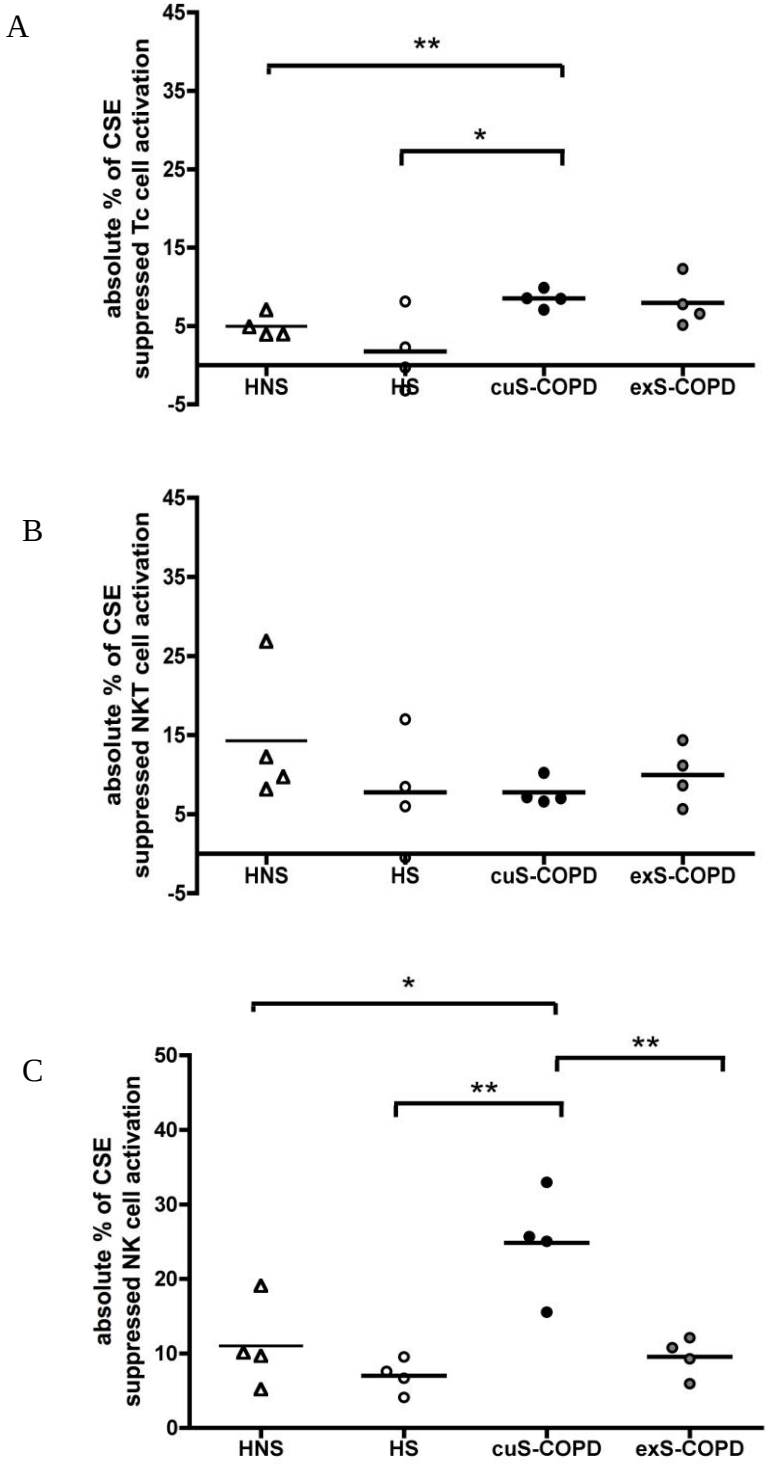



**Figure 3.2.11 Absolute suppression levels induced by CSE on IL-2 treated killer cell activation in four groups.** A) Tc cells; B) NKT cells; C) NK cells. HNS (healthy non-smokers, n=4); HS (current healthy smokers, n=4); C) cuS-COPD (current smokers with COPD, n=4); exS-COPD (ex-smokers with COPD, n=4). *, P<0.01; **, p<0.005; ***, p<0.001.

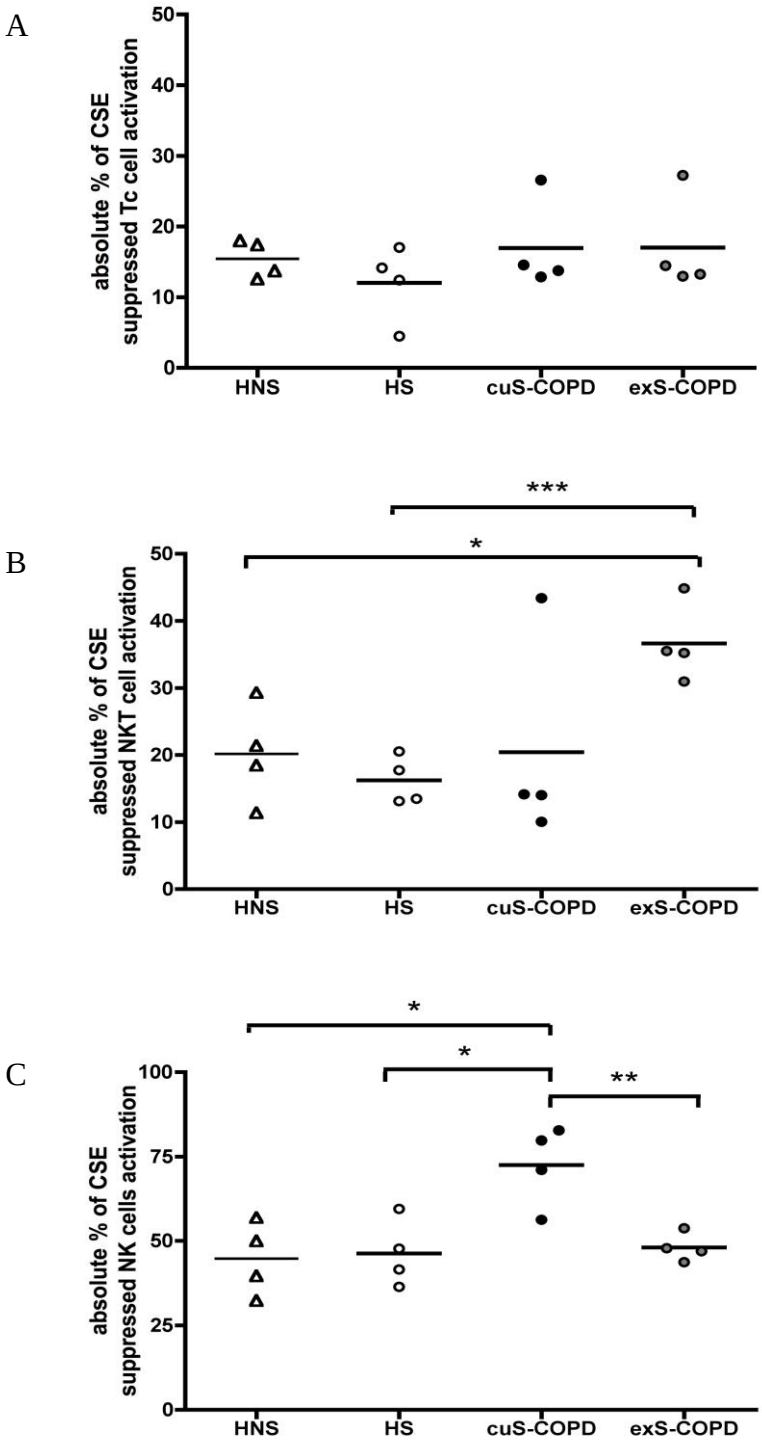



**Figure 3.2.12 Absolute suppression levels induced by CSE on IL-15 treated killer cell activation in four groups.** A) Tc cells; B) NKT cells; C) NK cells. HNS (healthy non-smokers, n=4); HS (current healthy smokers, n=4); C) cuS-COPD (current smokers with COPD, n=4); exS-COPD (ex-smokers with COPD, n=4). *, P<0.01; **, p<0.005; ***, p<0.001.

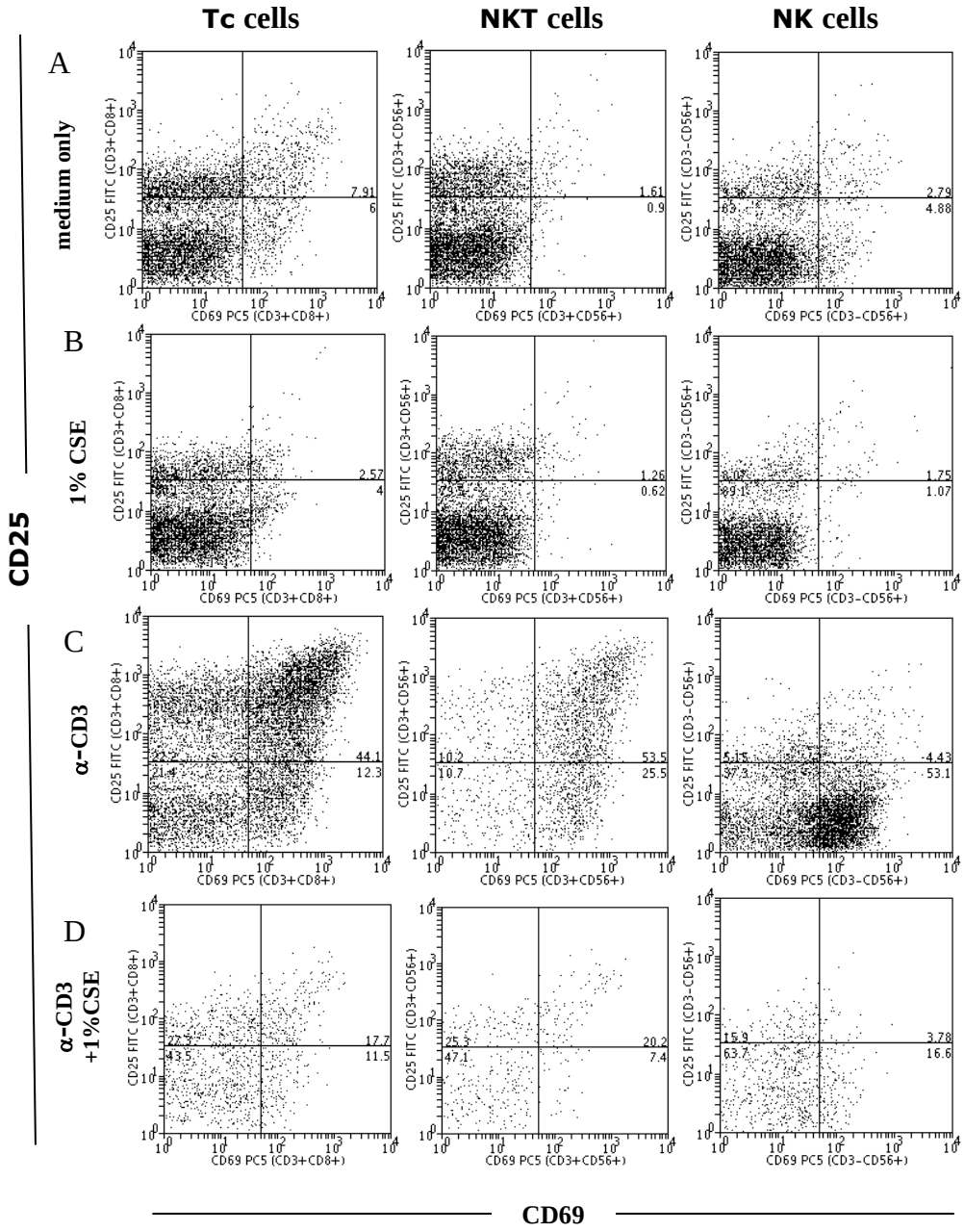
healthy non-smokers ($p<0.01$), current healthy smokers ($p<0.01$) and ex-smokers with COPD ($p<0.005$) (Figure 3.2.12.C).

Figure 3.2.13 showed the representative dot plots of CSE suppressed cell activation upon different activators from a current smoker with COPD. Figure1, Figure 2 and Figure 3 from Appendix showed the comparision of absolute activation levels of cells of interest upon different stimulation in four gourps. Table 3.2.8 showed the statistical differences of absolute suppression induced by CSE on different cell activation between groups.

### 3.2.4 Discussion

Cigarette smoking is still the major risk factor in the development of COPD. Accumulating evidence has suggested that chronic inhalation of cigarette smoke alters a wide range of immunologic function, including both innate and adaptive immune responses (Honda et al., 1996, Mio et al., 1997, Mohan, 2002b, Subramaniam et al., 1996, Wyatt et al., 1999). The main focus of this section was to investigate the impact of CSE on $\alpha$-CD3, IL-2 or IL-15 activated killer cells including Tc cells, NKT-like cells and NK cells, and further compared their activation levels in four groups, namely, healthy non-smokers,

current healthy smokers, current smokers with COPD and ex-smokers with COPD.



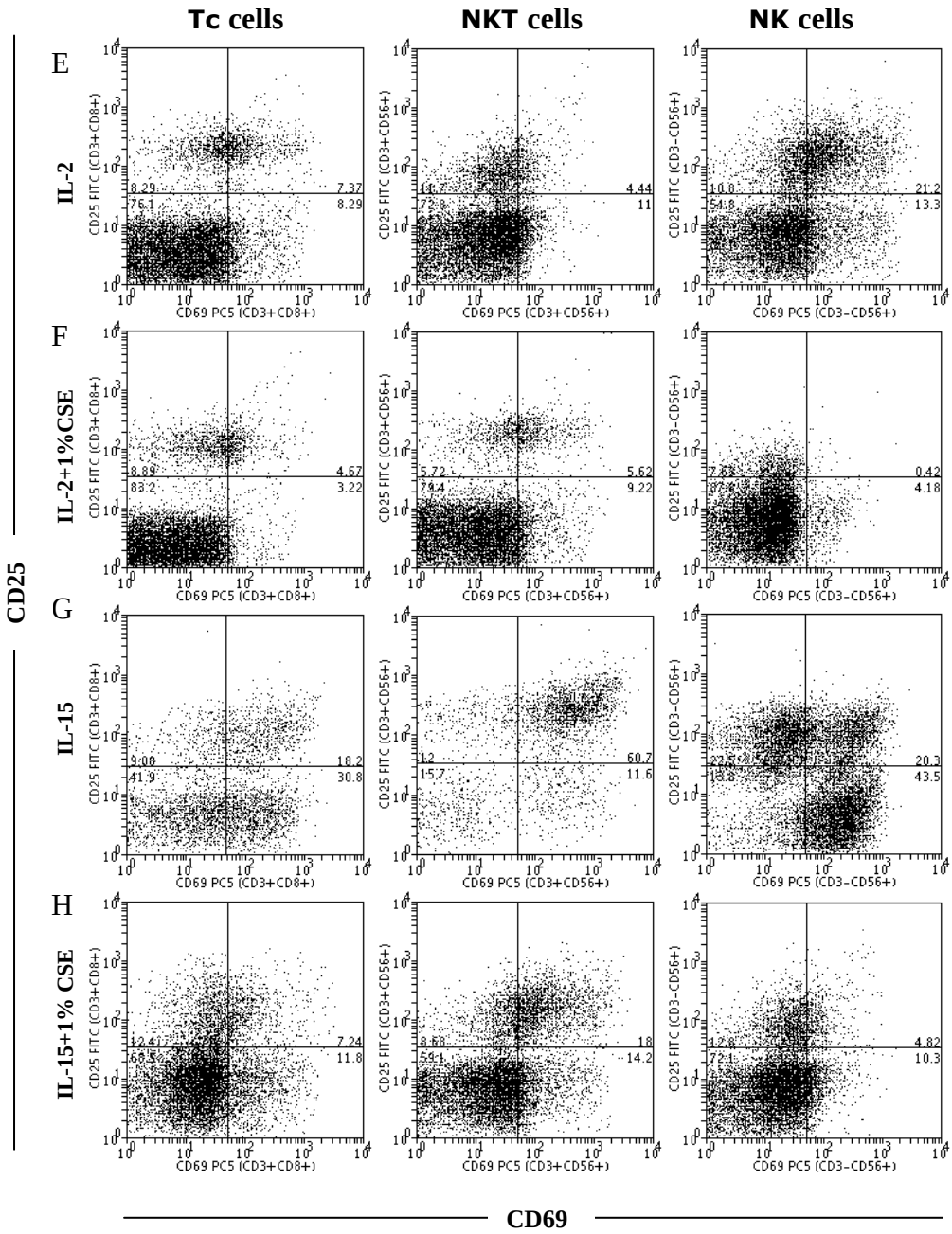



**Figure 3.2.13 Representative dot plots of the activation of interested cells from a current smoker with COPD.** PBMCs were stimulated by either A) meidium only; B) 1% CSE only; C) α-CD3; D) α-CD3+1% CSE; E) IL-2; F) IL-2+1% CSE; G) IL-15; or H) IL-15+1% CSE; and incubated for 48 hours after stimulation. α-CD3 significantly activated both Tc cells and NKT cells compared to NK cells. Whereas, IL-2 and IL-15 showed remarkable effects on NK cell activation.

**Table 3.2.8 Absolute suppression induced by CSE on activation of killer cells upon different stimulation.**

| | HNS vs HS | HNS vs cuS-COPD | HNS vs exS-COPD | HS vs cuS-COPD | HS vs exS-COPD | cuS-copd vs exS-COPD |
|---|---|---|---|---|---|---|
| | | | **α-CD3 treated cells** | | | |
| **Tc** | p>0.01 | p>0.01 | p>0.01 | p>0.01 | p>0.01 | p>0.01 |
| **NKT** | p>0.01 | **p<0.005** | p>0.01 | **p<0.01** | p>0.01 | **p<0.005** |
| **NK** | **p<0.01** | **p<0.01** | p>0.01 | **p<0.005** | p>0.01 | p>0.01 |
| | | | **IL-2 treated cells** | | | |
| **Tc** | p>0.01 | **p<0.005** | p>0.01 | **p<0.01** | p>0.01 | p>0.01 |
| **NKT** | p>0.01 | p>0.01 | p>0.01 | p>0.01 | p>0.01 | p>0.01 |
| **NK** | p>0.01 | **p<0.01** | p>0.01 | **p<0.005** | p>0.01 | **p<0.005** |
| | | | **IL-15 treated cells** | | | |
| **Tc** | p>0.01 | p>0.01 | p>0.01 | p>0.01 | p>0.01 | p>0.01 |
| **NKT** | p>0.01 | p>0.01 | **p<0.005** | p>0.01 | **p<0.001** | p>0.01 |
| **NK** | p>0.01 | **p<0.005** | p>0.01 | **p<0.01** | p>0.01 | **p<0.005** |

HNS, healthy non-smokers; HS, current healthy smokers; cuS-COPD, current smokers with COPD; exS-COPD, ex-smokers with COPD.

Firstly, to achieve optimal activation of all cells of interest, we set up a preliminary protocol to decide the optimal concentrations of each activator and incubation time for cell culture. Tc cells, NKT-like cells and NK cells were all highly activated when the concentration of α-CD3 increased to 1ug/ml. CD3 is a cell surface marker for T cells including Tc cells, Th cells, Treg cell, γ/δ T cells and NKT cells (Call and Wucherpfennig, 2004). However, in preliminary experiments, dose-dependent increases in the activation levels of NK cells were also observed, although NK cells do not express CD3/TCR complex on their cell surface. To our knowledge, this

could be easily explained. α-CD3 has the capacity of activating $CD4^+$ T cells, Tc cells and NKT cells that further release immune mediators such as IL-2 or IL-15 (Smyth et al., 2005, Thompson et al., 1989). Furthermore, previous studies have shown that CSE also has effects on DCs and macrophages that also secret high amount of IL-15 (Carson et al., 1995, Jonuleit et al., 1997). Both IL-2 and IL-15 are good sources for NK cell activation (Waldmann, 2006).

Activation of killer cells induced by IL-2 is not as great as IL-15 treated cells in healthy non-smokers. One of the reasons is the biological difference on features between IL-2 and IL-15. Although IL-2 and IL-15 share two receptors therefore have certain common features in their functions, they also provide distinct contributions on cell activation and apoptosis (Thomas, 2008, Waldmann and Tagaya, 1999). IL-2Rα is only expressed by pre B cells, effector T cells and DCs, whereas IL-15Rα is widely expressed on many cell types except regulatory T cells. Furthermore, IL-15 increases the proliferation of the $CD8^+$ T cells and reduces their death by apoptosis (Ku et al., 2000, Zhang et al., 1998), whereas IL-2 plays a critical role in Fas-mediated activation-induced cell death (Lenardo, 1996). Kanegane and Tosato has shown that in PBMCs from healthy volunteers, IL-2 and IL-15 can both activate memory $CD4^+$ T

cells, memory $CD8^+$ T cells and naive $CD8^+$ T cells, but not naive $CD4^+$ T cells (Kanegane and Tosato, 1996). In addition, IL-2 does not act on resting NKT cells or NK cells that without IL-2R$\alpha$ expression (Waldmann, 2002). We controlled the maximal concentration of IL-2 at 100ng/ml because of the side effects caused by high dose IL-2 administration as previously mentioned.

In our study, activation levels of killer cells were not significantly different in the absence or presence of CSE at increasing dilutions in healthy volunteers, without other stimulations. These findings were in agreement with other groups who detected the CSE effects on DCs or NK cells from healthy volunteers (Mian et al., 2009, Mortaz et al., 2009). Although Stringer *et al* showed that CSE impaired neutrophil caspase-3-like activity in a dilution-dependent manner (CSE dilution 2%-16%), they were concerned about different cell types and the incubation time was only for 4 hours (Stringer et al., 2007). According to previous studies and our detection, we selected 1% of CSE for further experiments because it did not cause any toxic effects as revealed by MMT assay (Mian et al., 2008, Mian et al., 2009, Stringer et al., 2007).

The effects of CSE on killer cells were explored not only in healthy volunteers but also COPD patients. Although CSE alone did not show any impacts on the killer cells activation in all interested groups, we here for the first time showed that CSE significantly reduced the activation levels of Tc cells, NKT cells and NK cells in response to $\alpha$-CD3, IL-2 or IL-15 in different groups (Table 3.2.7). Of note, three representative results should be pointed out. Firstly, CSE markedly suppressed activation levels of IL-15 treated all cell types in all groups. Secondly, in contrast to Tc cells and NKT cells, NK cells were the only cell type which activation levels upon different stimulation were all significantly decreased when CSE was added in all participant groups. Thirdly, current smokers with COPD were the only participant group that showed CSE had statistically significant effects on all killer cell type activation with the different stimulations. These findings were supported by different studies. Mian and colleague have shown that in PBMCs from healthy non-smokers, the expression of CD69, a key activation marker for NK cells, was significantly inhibited when PBMCs are treated by cigarette smoke after poly I:C activation (Mian et al., 2009). Also, the cigarette smoke markedly reduced NK cell cytolytic potential to kill K562 cancer cells which was found to be IL-15 mediated. Recently, Hogan *et al* demonstrated that CSE significantly

reduced the cytokine production and the ability to kill target cells of α-GalCel activated invariant NKT cells in PBMCs from healthy non-smoking participants. Meanwhile, the activation marker of CD107, but not CD69, was decreased (Hogan et al., 2011). Currently there are no previous studies to show the CSE effects on anti-CD3 activated killer cells.

Since CSE showed suppression effects on killer cells at different levels in each group, we further compared suppression levels in each cell type between healthy volunteers and patient groups. Due to the diverse background of each sample, we used absolute suppression levels to find out whether they have statistical differences. There were no significant differences of CSE suppression on Tc cells in response to α-CD3 or IL-15 between all groups. Interestingly, the absolute suppression levels induced by CSE on IL-2- or IL-15-treated NK cell activation were significantly higher in current smokers with COPD compared to other three groups, namely, healthy non-smokers, current healthy smokers and ex-smokers with COPD. In α-CD3 stimulated NKT cells, their activation levels were also dramatically suppressed when CSE was added in current smokers with COPD compared to healthy pariticipants and ex-smokers with COPD. However, CSE remarkably reduced the activation levels of NKT cells upon IL-

15 activation in ex-smokers with COPD, but not current smokers with COPD, compared to healthy non-smokers and current healthy smokers.

Chronic exposure to cigarette smoke leads to not only cellular death and damage in the airways, but also failure to activate critical intracellular signalling mechanisms that initiate protective host defence. The consequence is dysfunction of various cell types with accompanying predisposition to respiratory infection (Feldman and Anderson, 2013). Although small sample size was studied, our findings showed that in different conditions, CSE indeed suppressed killer cell activation in either healthy groups or COPD patients, or all of the samples. We believe in future, more reliable and consistent findings will be revealed when the sample size is enlarged.

It is important to note that cigarette smoke comprises a complex mixture of more than 4000 chemicals, such as nicotine, carbon monoxide, and tar (Hoffmann and Wynder, 1986, Stedman, 1968). Their immune-modulation effects on immune cells are distinct and also dose-dependent (Holt et al., 1976). To determine the specific component of cigarette

smoke that regulates killer cell activation upon IL-15 stimulation is beyond the scope of our studies.

In conclusion, we here for the first time demonstrate that absolute suppression levels induced by CSE on IL-2- or IL-15-mediated NK cell activation were significantly higher in current smokers with COPD patients compared to healthy non-smokers, current healthy smokers and ex-smokers with COPD. These findings provide further insights on understanding the cellular mechanisms of COPD and may also explain why smokers are more susceptible to lung infections.

## 3.3 Activation and cytotoxic molecule expression of $CD56^+$ cells from peripheral blood samples

### 3.3.1 Introduction

The previous chapter demonstrated that CSE significantly suppressed NK cell activation in IL-15 treated PBMCs, especially in COPD patients. Activation levels of NKT-like cells upon IL-15 stimulation were also markedly decreased in ex-smokers with COPD. Collectively, these findings suggested that CSE had impact on IL-15 mediated $CD56^+$ cell activation.

One of the major killing mechanisms of cytotoxic cells is performed by killing molecules, such as granzymes and perforin, and Fas-Fas ligand (Fairclough et al., 2008). After recognition of target cells by effector cells, granules released from killer cells move to the immunological synapse by exocytosis. Their membrane then fuses with the killer cell plasma membrane and releases contents into target cells. Previous studies have shown that increased expression of perforin and granzyme B were detected on $CD8^+$ T cells from BAL fluid in COPD patients (Hodge et al., 2006). The percentage of both granzyme B and perforin positive NKT-like

and NK cells were significantly higher in induced sputum from COPD patients (Urbanowicz et al., 2010), but lower in peripheral blood samples (Urbanowicz et al., 2009). Morissette and colleague found that the percentage of peripheral $CD8^+$ T cells and $CD56^+$ cells expressing perforin, granzyme B and Fas-ligand was not different between healthy non-smokers, healthy smokers and emphysematous smokers (Morissette et al., 2007). However, it is hard to conclude that perforin and granzyme B positive NKT cells are the critical difference between COPD and emphysematous conditions. More studies should be carried out to confirm it. Furthermore, up-regulated soluble Fas was observed in plasma from severe COPD patients but not mild or moderate COPD patients (Yasuda et al., 1998).

In PBMCs, IL-15 not only stimulates NK cells or NKT cells, but also other immune cells, such as memory $CD4^+$ T cells and $CD8^+$ T cells, which consequently secrete more functional cytokines and chemokines to induce adaptive immune response (Anderson et al., 1995, Cosman et al., 1995). To confirm whether the suppression effects caused by CSE were directly related to IL-15 treated NKT-like cells and NK cells, in this section, we isolated NKT-like cells ($CD3^+CD56^+$) and NK cells ($CD3^-CD56^+$) from PBMCs in healthy non-smoker, current

healthy smokers and current smokers with COPD. Both activation levels and killing molecule expression were investigated and compared in three groups. Two types of cells were incubated with medium alone, CSE alone or IL-15 in the absence or presence of CSE, for 48 hours. Cell activation markers CD69 and CD25 were measured, along with killing molecules including perforin, granzyme B and Fas-ligand.

### 3.3.2 Material and methods

#### 3.3.2.1 Study population and procedures

The criteria of participant enrolments are the same as previously introduced. Nine participants were included in this part of the study. Table 3.3.1 shows the demographic and spirometric data of the participants.

#### 3.3.2.2 Reagents

Recombined human IL-15 was obtained from PeproTech EC (London, UK). Other monoclonal antibodies for flowcytometry were shown in table 3.3.2.

#### 3.3.2.3 Cigarette smoke extract preparation

CSE were freshly prepared prior to 30 minutes of cell culture for each experiment to avoid breakdown of substances in the

**Table 3.3.1 Demographic and spirometric valus of the CSE suppression study groups**

| | Healthy non-smokers | Current healthy smokers | cuS-COPD |
|---|---|---|---|
| **Participants** | 3 | 3 | 3 |
| **Age (years)** | 52 (50-54) | 57 (54-60) | 55 (52-58) |
| **Gender (M/F)** | 0/3 | 2/1 | 2/1 |
| **Packs/yrs** | 0 | 39 (31-42) | 43 (35-47) |
| **Smoking status (Current/Ex)** | 0 | 3/0 | 3/0 |
| **Chronic bronchitis (Yes/No)** | 0 | 2/1 | 3/0 |
| **$FEV_1$ (% pred)** | 111 (102-119) | 105 (98-117) | 52 (46-60) |
| **$FEV_1$/FVC (%)** | 97 (86-113) | 90 (69-106) | 49(45-53) |
| **$\Delta FEV_1$ post bronch** | 2.2 (1.7-2.5) | 2.0 (1.7-2.4) | 1.5 (1.2-1.6) |
| **BMI ($kg/m^2$)** | 25.8 (22.6-28.3) | 26.7 (20.5-29.4) | 20.3 (15.8-25.1) |
| **Inhaled GCS (on/off)** | N/A | N/A | 0/3 |
| **MRC dyspnoea scale** | N/A | N/A | 3 (2-4) |
| **Distance walked in 6 min (m)** | N/A | N/A | 277 (196-312) |
| **BODE Index** | N/A | N/A | 4 (3-6) |

extract and evaporation of volatile components. More details are provided in section 2.4.

### 3.3.2.4 Separation of NKT-like cells and NK cells

Isolated PBMCs were incubated with anti-CD56 MicroBeads (Miltenyi Biotech Ltd, Surrey, UK) in MACS buffer containing sterile D-PBS (Sigma), 1% heat-inactivated fetal calf serum (FCS) (QB-Perbio) and 0.4% EDTA (Sigma) for 15 minutes at 4°C. More details are provided in section 2.3.

**Table 3.3.2 Antibodies used for flow cytometry**

| Antigen | Fluorochrome | Isotype | Clone | Source |
|---|---|---|---|---|
| CD3 | PC7 | Mouse IgG1 | UCHT1 | Beckman Coulter, Luton, UK |
| CD25 | PE | Mouse IgG2a, | B1.49.9 | Beckman Coulter, Luton, UK |
| CD56 | PC5 | Mouse IgG1 | N901 | Beckman Coulter, Luton, UK |
| CD69 | ECD | Mouse IgG2b | TP1.55.3 | Beckman Coulter, Luton, UK |
| KIR3DL1 (CD158e1) | FITC | Mouse IgG1, k | DX9 | Biolegend, San Diego, CA, USA |
| Fas-L (CD178) | PE | IgG Hamster | 4A5 | Beckman Coulter, Luton, UK |
| GranzymeB | FITC | Mouse IgG1, k | GB11 | BD Pharmingen™ |
| Perforin | PE | Mouse IgG2b, k | δG9 | BD Pharmingen™ |

### 3.3.2.5 Cell culture and treatments

Isolated NKT-like cells and NK cells were separately resuspended in RPMI-1640 medium supplemented with 10% of FCS at a density of $1x10^6$ cells/ml and cultured at 200ul/well in a 96-well flat bottom tissue culture plate. These cells were either untreated or treated with CSE, IL-15 or IL-15 plus CSE. All cells were cultured at 37°C in a 5% $CO_2$ for 48 hours before analysis.

#### 3.3.2.6 Extracellular staining for $CD56^+$ cells

Fluorescently conjugated antibodies used for extracellular staining were as follows, CD3 (PC7), CD56 (PC5), CD69 (ECD) CD25 (PE) and Fas-L (PE), or KIR (FITC). More details are shown in section 2.6.

#### 3.3.2.7 Intracellular staining for $CD56^+$ cells

Fluorescently conjugated antibodies including perforin (PE) and granzyme B (FITC) were used for intracellular staining. Briefly, Brefeldin A (Sigma, 0.4ul for per $2\times10^5$ cells) diluted in RPMI medium was added to cell culture prior to 14 hours before harvesting cells. Cell suspension were washed with PBA, fixed with 3% of formaldehyde fix and incubated in the dark at 4°C for 5 minutes. More details are shown in section 2.6.

#### 3.3.2.8 Flow cytometric analysis

Flow cytometric analysis of antibody labelled cells was performed using an EPICS Altra (Beckman Coulter, Luton, UK). More details are provided in section 2.9.

#### 3.3.2.9 Statistical analysis

The statistical analysis was performed with Prism software, version 4.0c (GraphPad). Differences between the three groups of participants were tested using the parametric

student's *t* test. Confidence interval 99% was used to show two-tailed p values. P values of less than 0.01 were considered to indicate statistical significance.

### 3.3.3 Results

#### 3.3.3.1 Demographic and medical characteristics of COPD participants, healthy smokers and healthy controls

There were no statistical differences between groups in terms of age or pack years smoked between current smokers with COPD and current healthy smokers (Data not shown).

#### 3.3.3.2 Effects of CSE on $CD56^+$ cell activation

Isolated NKT-like cells and NK cells were incubated with medium alone, CSE (1%) alone, IL-15 (20ug/ml) in the absence or in the presence of CSE (1%) for 48 hours at 37°C before cell collection. Cells are designated activated when they express one or both activation markers (i.e., CD69 positive, CD69/CD25 double positive or CD25 positive). There were no significant differences on the activation levels of NKT-like cells (Figure 3.3.1.A) or NK cells (Figure 3.3.1.C) between medium and CSE alone incubation in all groups (all $p>0.01$). As shown in figure 3.3.1.B, activation levels of CSE treated NKT-like cells upon IL-15 stimulation were significantly suppressed

compared to IL-15 itself mediated cell activation in all groups ($p<0.01$). Also, CSE very markedly suppressed IL-15 mediated NK cell activation in all groups (Figure 3.3.1.D; HNS, $p<0.005$; HS, $p<0.005$; cuS-COPD, $p<0.001$). Figure 3.3.2 shows the representative dot plots of CSE treated NKT-like cell and NK cell activation upon IL-15 stimulation in current smokers with COPD.

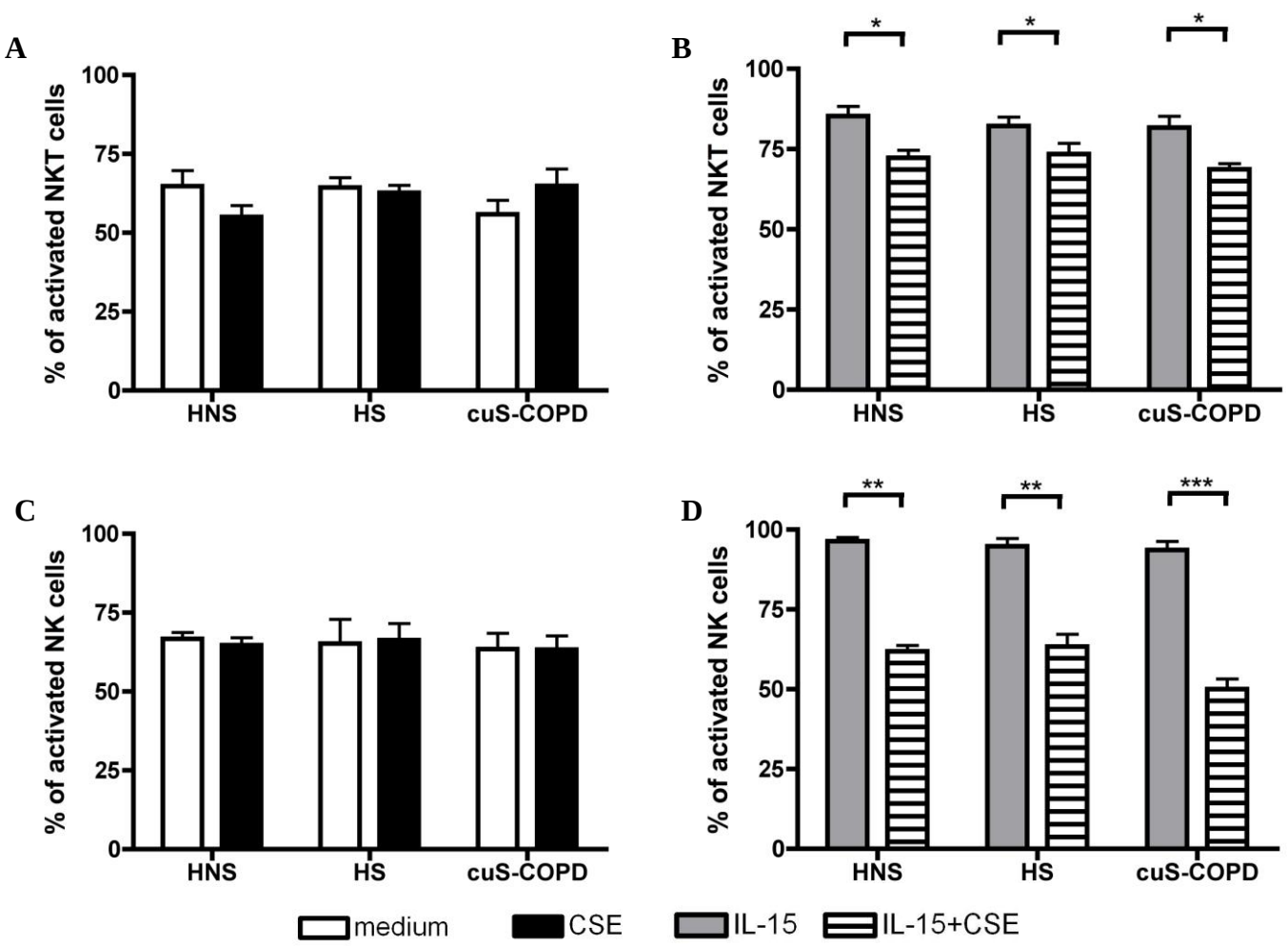



**Figure 3.3.1 CSE effects on activation levels of NKT-like cells and NK cells in different conditioned medium.** NKT-like cells (panel A and B) and NK cells (panel C and D) were isolated from PBMCs in three groups including health non-smokers (HNS, n=3), current current healthy smokers (HS, n=3), and current smokers with COPD (cuS-COPD, n=3). Cells were untreated or treated by CSE alone, IL-15 alone or IL-15+CSE and incubated 48 hours. Significant differences were considered as p value<0.05. *, p<0.01; **, p<0.005; ***, p<0.001. Cells are designated activated when they express one or both activation markers including CD69 and CD25.

### 3.3.3.3 Effects of CSE on killing molecule expression by $CD56^+$ cells

Killing molecules, such as granzymes and perforin, and Fas-Fas ligand, perform major functions in cytotoxic cell mediated immune responses. Here we detected, *in vitro*, the effects of CSE on expression of granzyme B (Figure 3.3.3), perforin (Figure 3.3.4), and Fas-ligand (Figure 3.3.5) in NKT-like cells and NK cells upon IL-15 stimulation. There were no significant differences on the expression of granzyme B by either NKT cells or NK cells that were treated with or without CSE (Figure 3.3.3.A and Figure 3.3.3.C). The expression of granzyme B on IL-15-activated NKT-like cells were significantly inhibited by CSE in healthy non-smokers ($p<0.01$) (Figure 3.3.3.B). Also, CSE dramatically reduced the expression of granzyme B on IL-15-treated NK cells in both healthy non-smokers (Figure 3.3.3.D, $p<0.01$) and current smokers with COPD (Figure 3.3.3.D, $p<0.001$). Perforin was highly expressed in both NKT-like cells and NK cells with or without extra stimulation in all groups (Figure 3.3.4). Whereas Fas-ligand expressed by NKT-like cells and NK cells was lower in all cell incubation conditions in three studied groups (Figure 3.3.5). Table 3.3.3 and table 3.3.4 showed the actual value range of expression levels of molecules of interest on NKT cells and NK cells.

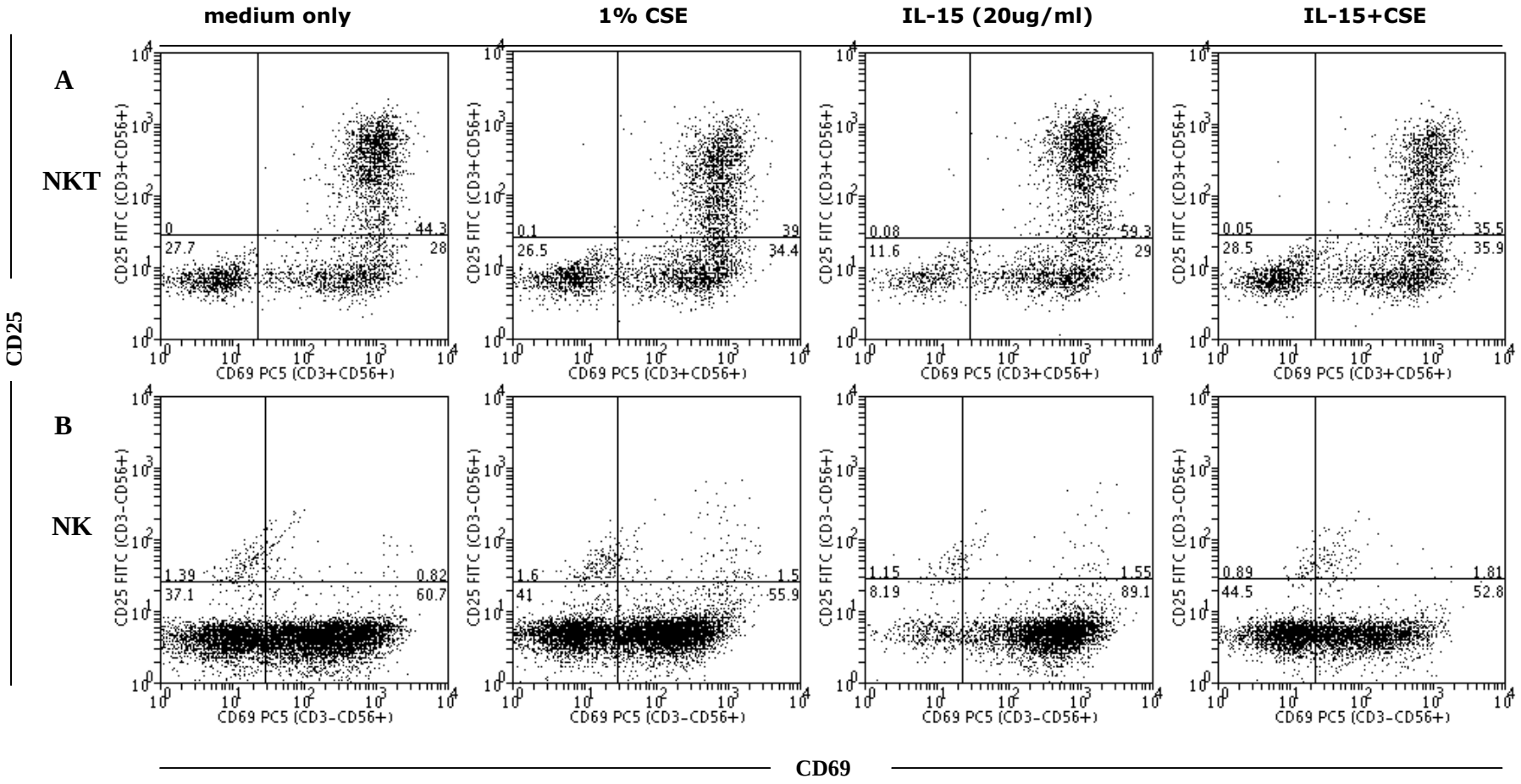



**Figure 3.3.2 Representative dot plots for activation of NKT-like cells (panel A) and NK cells (panel B) isolated from PBMCs from a current smoker with COPD.** NKT-like cells or NK cells were either untreated or treated with CSE alone, IL-15 alone or IL-15 co-treated with CSE.

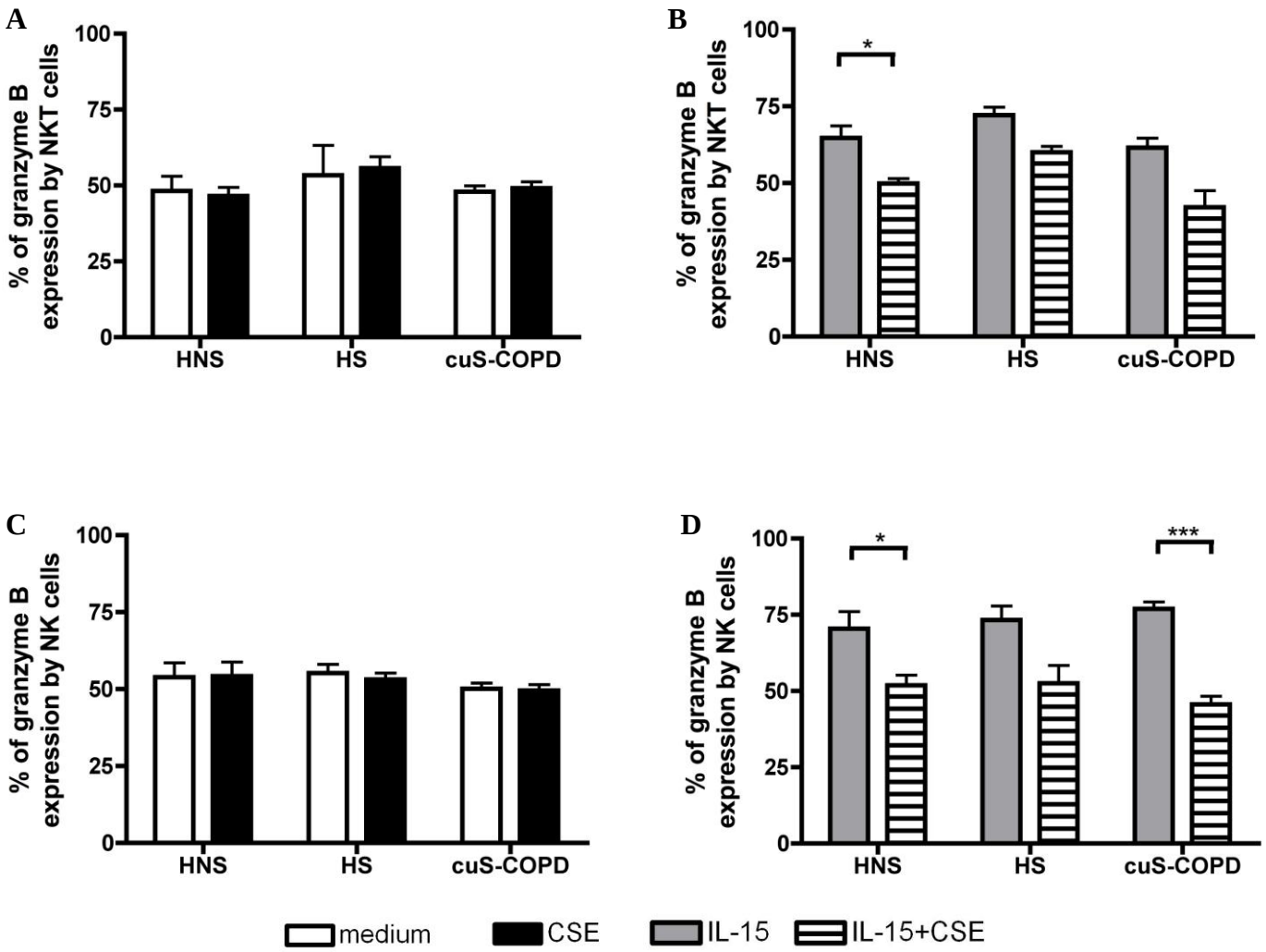



**Figure 3.3.3 CSE effects on expression of granzyme B by NKT-like cells and NK cells.** NKT-like cells (panel A and B) and NK cells (panel C and D) were isolated from PBMCs in three groups including health non-smokers (HNS, n=3), current healthy smokers (HS, n=3), and current smokers with COPD (cuS-COPD, n=3). Cells were untreated or treated by CSE alone, IL-15 alone or IL-15+CSE and incubated 48 hours. *, $p<0.01$; **, $p<0.005$; ***, $p<0.001$.

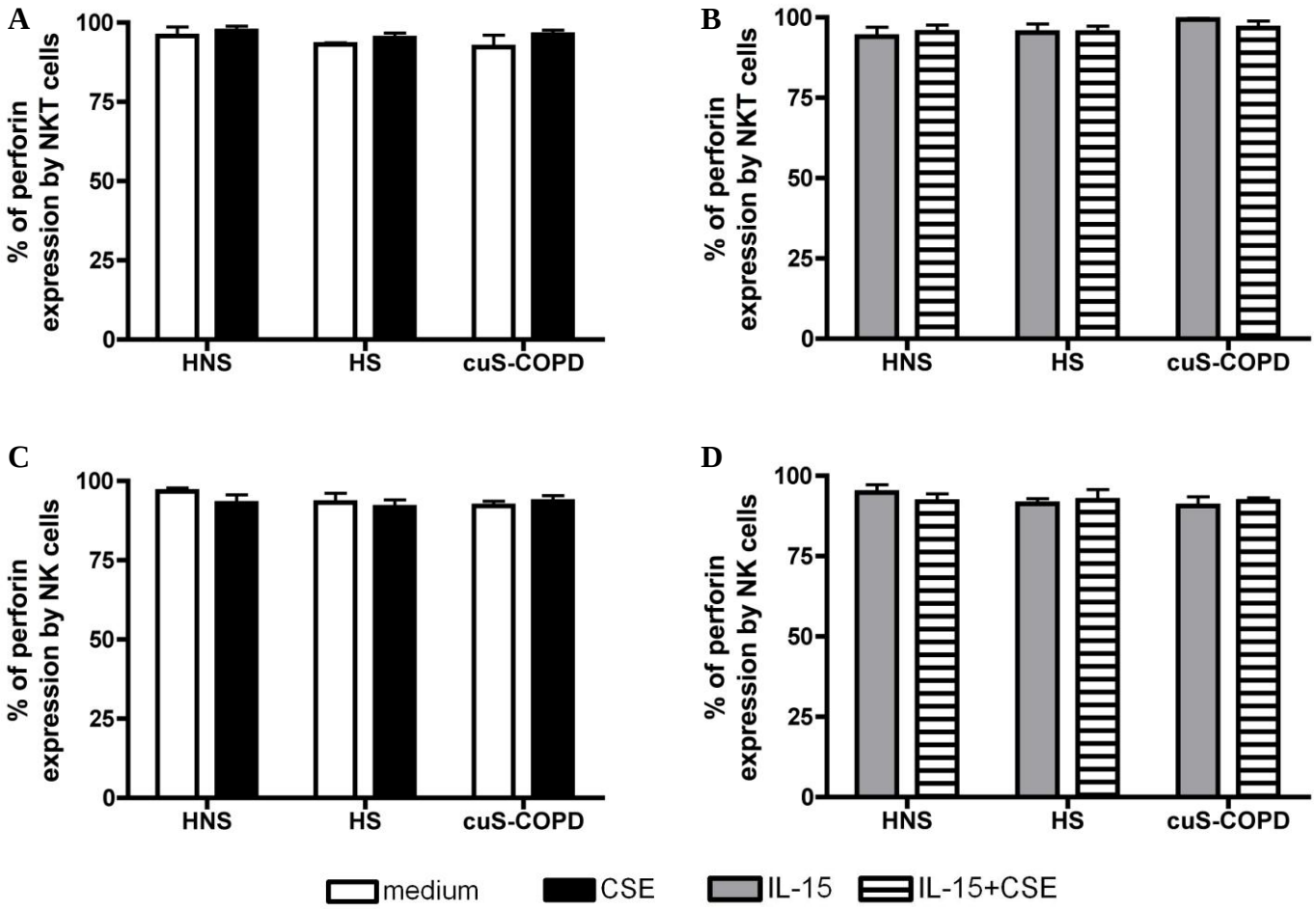



**Figure 3.3.4 CSE effects on expression of perforin by NKT-like cells and NK cells.** NKT-like cells (panel A and B) and NK cells (panel C and D) were isolated from PBMCs in three groups including health non-smokers (HNS, n=3), current healthy smokers (HS, n=3), and current smokers with COPD (cuS-COPD, n=3). Cells were untreated or treated by CSE alone, IL-15 alone or IL-15+CSE and incubated 48 hours. *, $p<0.01$; **, $p<0.005$; ***, $p<0.001$.

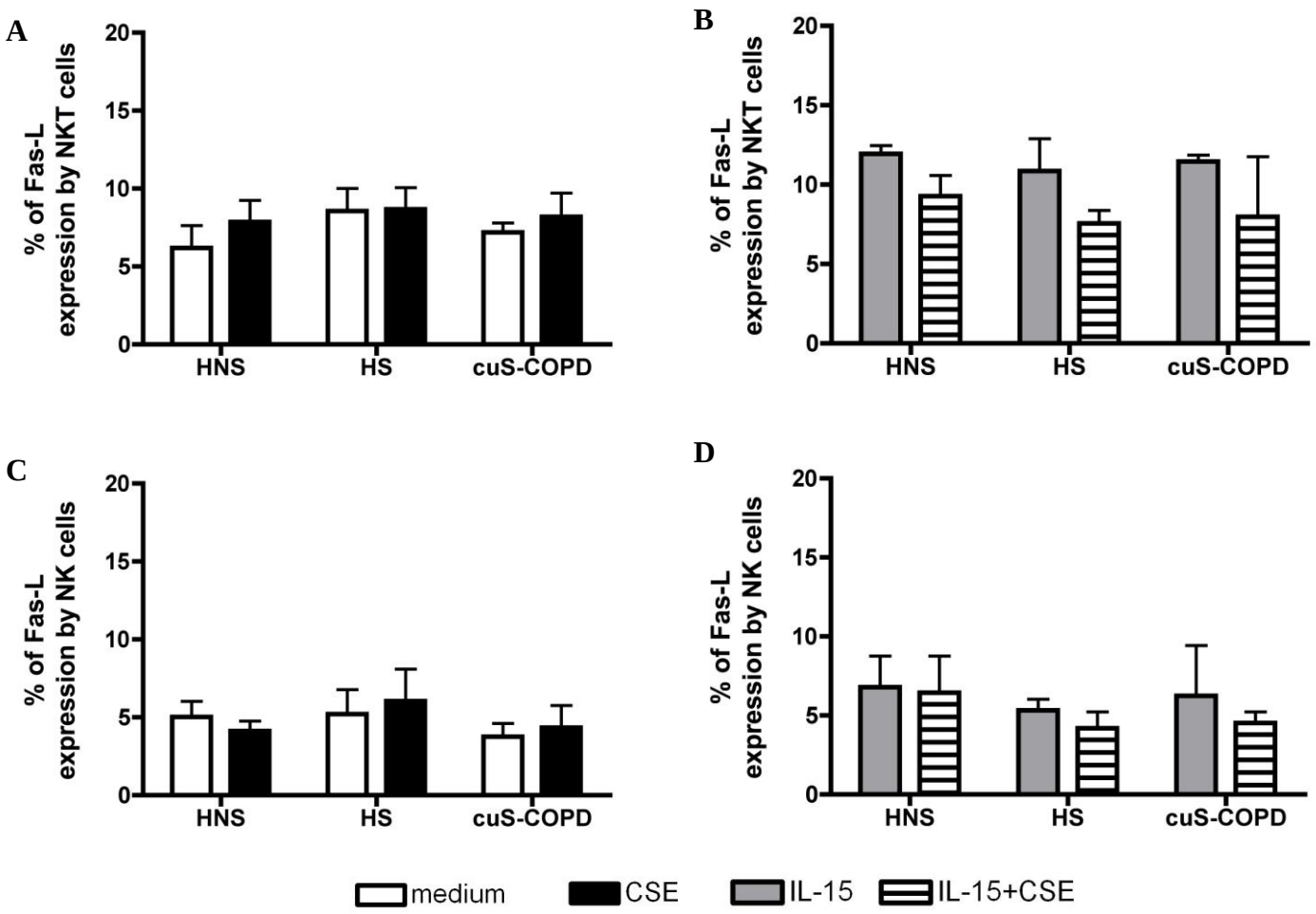



**Figure 3.3.5 CSE effects on expression of Fas-ligand by NKT-like cells and NK cells.** NKT-like cells (panel A and B) and NK cells (panel C and D) were isolated from PBMCs in three groups including health non-smokers (HNS, n=3), current healthy smokers (HS, n=3), and current smokers with COPD (cuS-COPD, n=3). Cells were untreated or treated by CSE alone, IL-15 alone or IL-15+CSE and incubated 48 hours. *, $p<0.01$; **, $p<0.005$; ***, $p<0.001$.

**Table 3.3.3 Expression of molecules of interest on NKT cells with or without activation.**

| **Granzyme B** | | | | |
|---|---|---|---|---|
| | Medium only | CSE only | IL-15 | IL-15 + CSE |
| **HNS** | 48.2% (39.2-55.6) | 46.6% (43.6-52.1) | 64.7% (57.8-71.3) | 49.8% (47.6-52.9) |
| **HS** | 53.3% (37.8-71.6) | 55.7% (50.0-62.0) | 72.1% (69.1-77.2) | 60.1% (56.8-63.3) |
| **CuS-COPD** | 47.9% (44.0-50.0) | 49.1% (45.1-52.2) | 61.5% (55.7-66.6) | 42.1% (33.7-52.2) |
| **Perforin** | | | | |
| | Medium only | CSE only | IL-15 | IL-15 + CSE |
| HNS | 95.8% (90.1-99.1) | 97.3% (94.2-99.2) | 94.0% （90.3-99.8） | 95.4% (91.2-98.8) |
| HS | 93.1% (92.3-94.0) | 95.1% (92.2-97.6) | 95.3% （90.3-99.0） | 95.3% (91.7-98.8) |
| CuS-COPD | 92.3% (87.2-99.6) | 96.2% (93.3-98) | 99.31% (98.2-99.9) | 96.7% (92.4-99.0) |
| **Fas-ligand** | | | | |
| | Medium only | CSE only | IL-15 | IL-15 + CSE |
| **HNS** | 6.2% (3.4-8.0) | 7.9% (5.6-10.3) | 12.0% (11.0-12.7) | 9.3% (7.2-11.7) |
| **HS** | 8.6% (5.7-10.2) | 8.7% (6.6-11.3) | 10.9% (7.3-14.4) | 7.6% (6.3-9.1) |
| **CuS-COPD** | 7.2% (6.1-8.1) | 8.2% (6.4-11.2) | 11.5% (11.0-12.3) | 8.0% (2.1-15.1) |
| **KIR** | | | | |
| | Medium only | CSE only | IL-15 | IL-15 + CSE |
| **HNS** | 7.9% (6.0-9.9) | 8.3% (5.1-10.1) | 9.0% (5.1-11.2) | 8.5% (7.3-9.3) |
| **HS** | 9.8% (7.4-12.1) | 9.3% (8.8-10.3) | 11.2% (8.2-15.2) | 7.3% (5.5-9.2) |
| **CuS-COPD** | 9.0% (8.9-9.1) | 9.7% (8.0-11.2) | 12.7% (10.3-15.1) | 6.8% (2.5-10.2) |

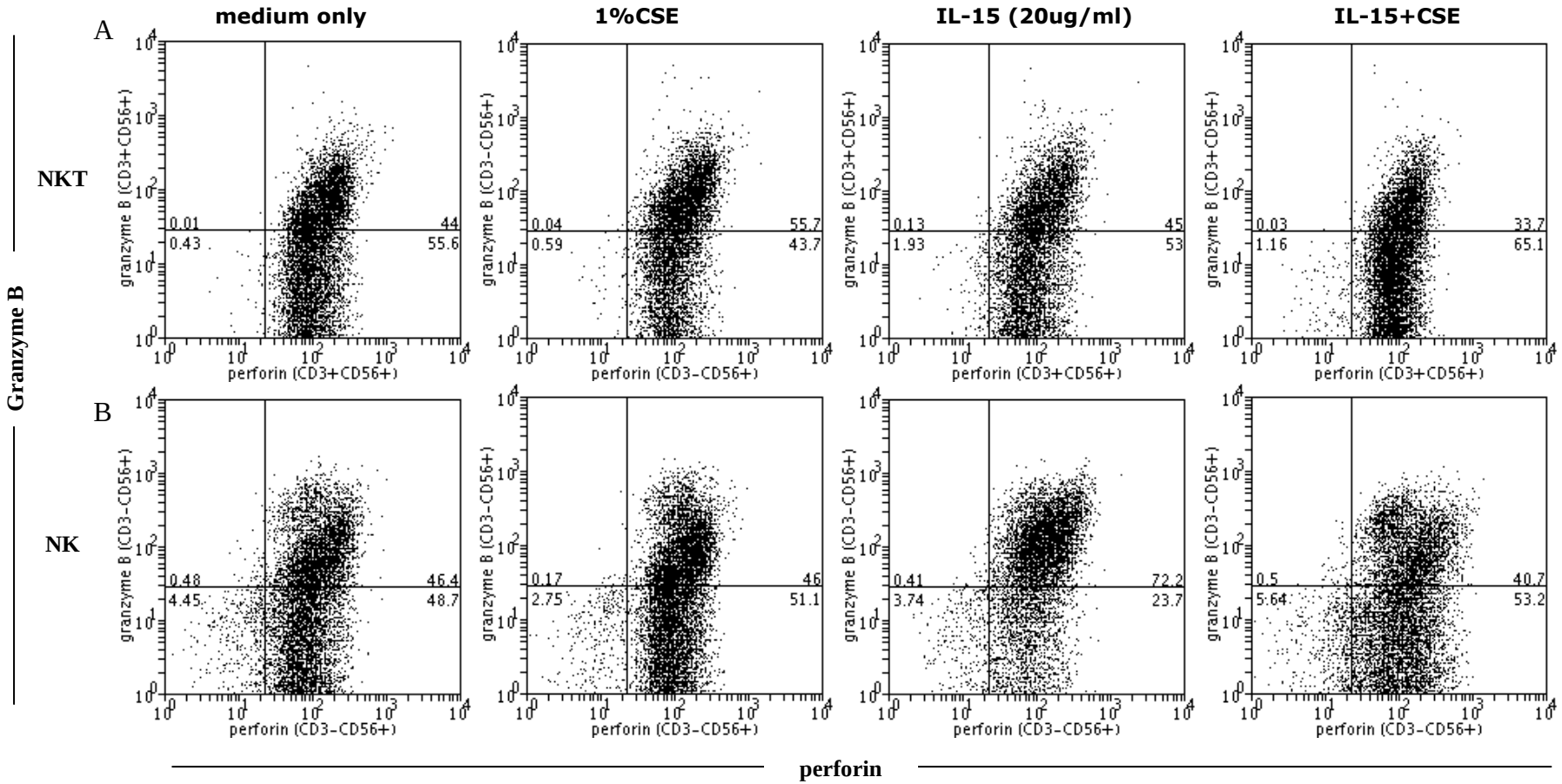
**Table 3.3.4. Expression of molecules of interest on NK cells with or without activation.**

| **Granzyme B** | | | | |
|---|---|---|---|---|
| | Medium only | CSE only | IL-15 | IL-15 + CSE |
| **HNS** | 53.9% (44.9-60.1) | 54.2% (45.2-59.3) | 70.5% (61.1-80.3) | 51.9% (46.5-57.9) |
| **HS** | 55.2% (50.1-60.1) | 53.1% (49.0-56.9) | 73.3% (67.0-82.2) | 52.6% (42.0-62.1) |
| **CuS-COPD** | 50.1% (46.9-53.4) | 49.6% (46.2-52.6) | 76.9% (72.6-80.3) | 45.7% (41.2-50.2) |
| **Perforin** | | | | |
| | Medium only | CSE only | IL-15 | IL-15 + CSE |
| HNS | 96.6% (95.5-98.7) | 92.9% (90.0-98.3) | 94.8% （90.0-97.7） | 91.9% (87.3-95.5) |
| HS | 93.2% (91.0-99.2) | 91.7% (87.9-95.9) | 91.3% （89.4-94.4） | 92.4% (88-98.9) |
| CuS-COPD | 92.1% (90.2-95.1) | 93.6% (90.9-97.1) | 90.6% (856.-95.5) | 92.1% (90.1-93.9) |
| **Fas-ligand** | | | | |
| | Medium only | CSE only | IL-15 | IL-15 + CSE |
| **HNS** | 5.0% (3.8-7.0) | 4.1% (3.0-5.1) | 12.0% (3.4-10.2) | 9.3% (3.4-11.0) |
| **HS** | 5.2% (2.3-7.7) | 6.0% (2.9-9.9) | 10.9% (3.9-6.2) | 7.6% (2.4-5.9) |
| **CuS-COPD** | 3.8% (2.2-5.1) | 4.3% (2.1-7) | 11.5% (0.3-11.2) | 8.0% (3.2-5.6) |
| **KIR** | | | | |
| | Medium only | CSE only | IL-15 | IL-15 + CSE |
| **HNS** | 5.8% (3.3-7.9) | 4.3% (0.9-9.1) | 5.0% (0.7-8.9) | 5.4% (2.5-10.2) |
| **HS** | 4.6% (2.1-7.2) | 3.1% (1.8-5.4) | 5.3% (2.9-7.6) | 4.3% (2.4-6.0) |
| **CuS-COPD** | 3.4% (1.5-5.7) | 2.1% (0.7-3.5) | 6.1% (0.6-10.1) | 2.6% (1.7-3.3) |

CSE had no effects on the expression levels of either perforin or Fas-ligand. Collectively, IL-15 had the ability of inducing more granzyme B expression on $CD56^+$ cells but had no effects on the expression of perforin and Fas-ligand. Figure 3.3.6 shows representative dot plots of CSE effects on killing molecule expression by $CD56^+$ cells from current smokers with COPD.

### 3.3.3.4 Effects of CSE on KIR expression by $CD56^+$ cells

The effect of CSE on KIR expression was also studied (Figure 3.3.7). Results in chapter 3.1 demonstrated that the expression of KIR (CD158e1), *in vivo*, was much more smoking-related than disease related (Figure 3.1.8). However, here, *in vitro,* we did not detect any statistical differences on KIR expression by CSE treated $CD56^+$ cells upon IL-15 stimulation KIR expression showed very low levels on $CD56^+$ cells in each group. Due to low cell numbers of $CD56^+$ cells, we did not detect the NKG2D expression.


medium only
1%CSE
IL-15 (20ug/ml)
IL-15+CSE
A
B
NKT
NK
Granzyme B
perforin
granzyme B (CD3+CD56+)
perforin (CD3+CD56+)
granzyme B (CD3-CD56+)
perforin (CD3-CD56+)
0.01
44
0.43
55.6
0.04
55.7
0.59
43.7
0.13
45
1.93
53
0.03
33.7
1.16
65.1
0.48
46.4
4.45
48.7
0.17
46
2.75
51.1
0.41
72.2
3.74
23.7
0.5
40.7
5.64
53.2

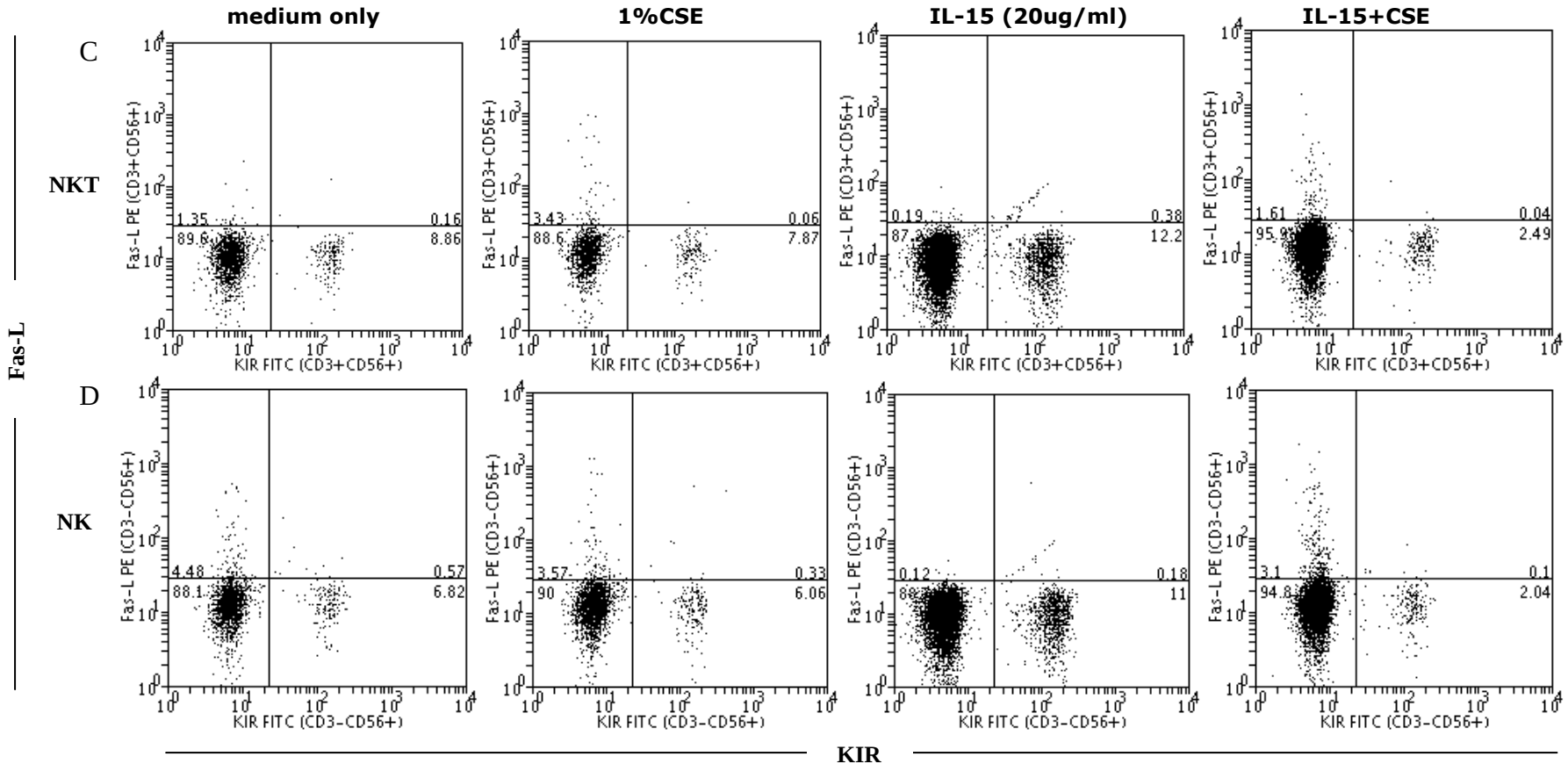



**Figure 3.3.6 Representative dot plots for expression of perforin, granzyme B, Fas-ligand and KIR by NKT-like cells (panel A and B) and NK cells (panel C and D) isolated from PBMCs from a current smoker with COPD.** NKT-like cells or NK cells were either untreated or treated with CSE alone, IL-15 alone or IL-15 co-treated with CSE.

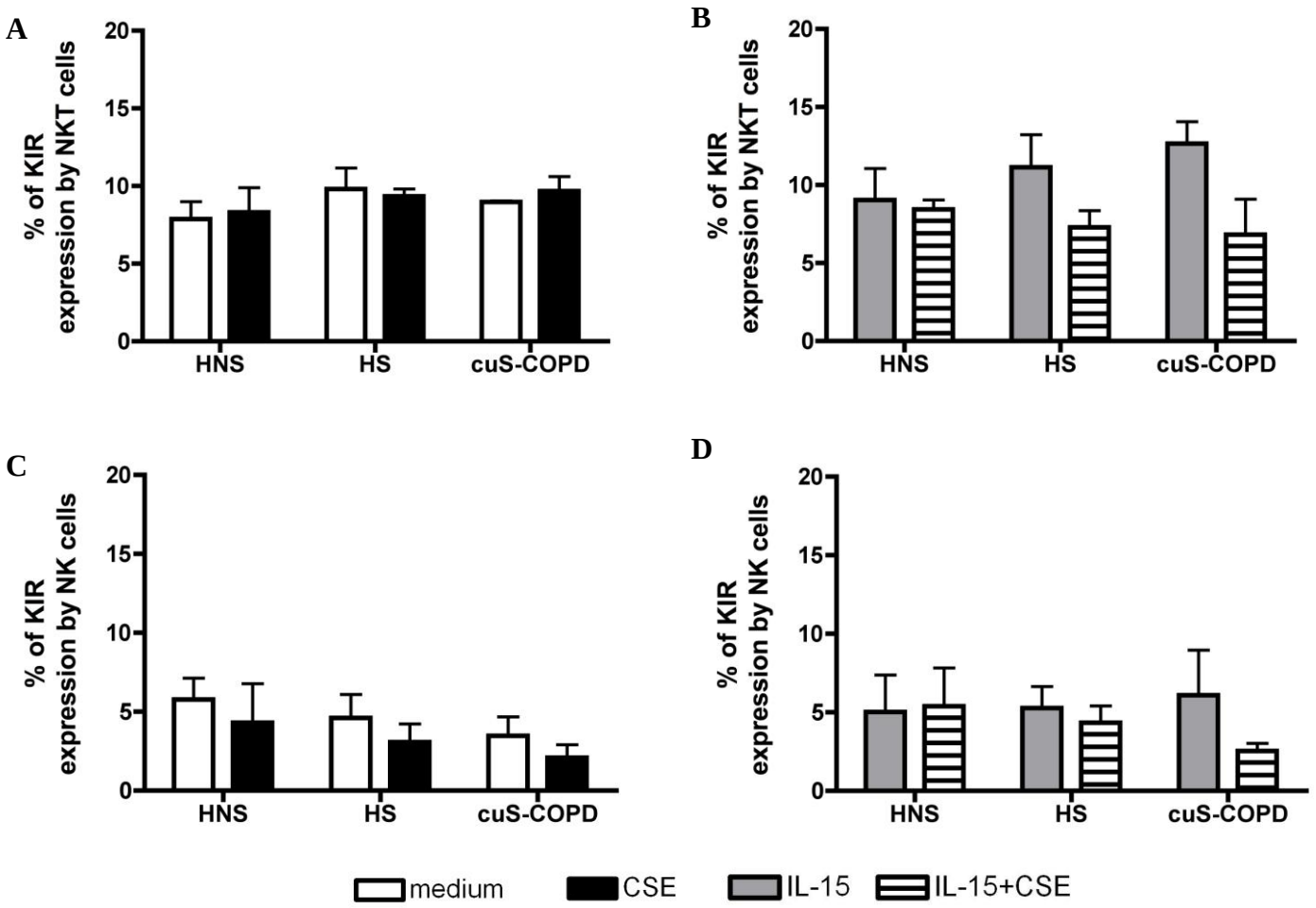



**Figure 3.3.7 CSE effects on expression of KIR by NKT-like cells and NK cells.** NKT-like cells (panel A and B) and NK cells (panel C and D) were isolated from PBMCs in three groups including health non-smokers (HNS, n=3), current healthy smokers (HS, n=3), and current smokers with COPD (cuS-COPD, n=3). Cells were untreated or treated by CSE alone, IL-15 alone or IL-15+CSE and incubated 48 hours. *, $p<0.01$; **, $p<0.005$; ***, $p<0.001$.

### 3.3.3.5 CSE suppressed cell activation

As previously mentioned, cell activation was measured by expressing one or both of the activation markers including CD69 and CD25. CSE significantly suppressed the activation levels of IL-15 treated NKT-like cells and NK cells. Here we compared the absolute suppression induced by CSE on IL-15 mediated NKT-like cell or NK cell activation in three groups. There were no statistical differences on the suppression levels between groups (Figure 3.3.8.A and B). The expression levels of granzyme B on CSE treated NKT-like cells or NK cells in response to IL-15 were also compared. No significant differences were detected on the absolute suppression of granzyme B expression between groups (Figure 3.3.9.A and B).

## 3.3.4 Discussion

Here we report, for the first time, *in vitro*, that CSE significantly suppresses the activation levels of IL-15 treated NKT-like cells and NK cells isolated from PBMCs in healthy non-smokers, current healthy smokers and current smokers with COPD.

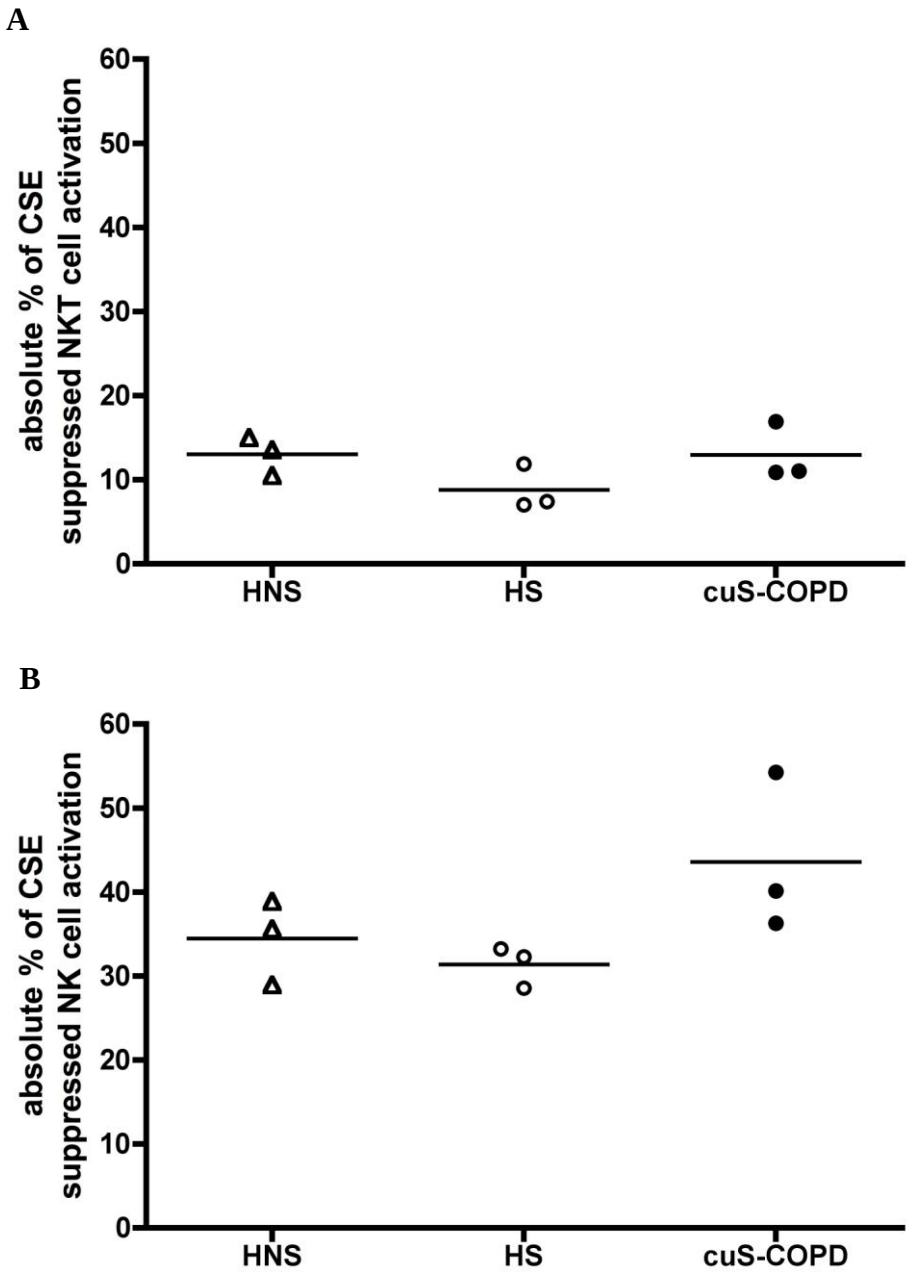



**Figure 3.3.8 CSE induced suppression on activation levels of IL-15 stimulated NKT-like cells and NK cells in three groups.** Absolute suppression levels were gained by using (% of IL-15 activated cells) minus (% of IL-15+CSE treated cells). All p value>0.05. HNS (healthy non-smokers, n=3); HS (healthy smokers, n=3); cuS-COPD (current smokers with COPD, n=3).

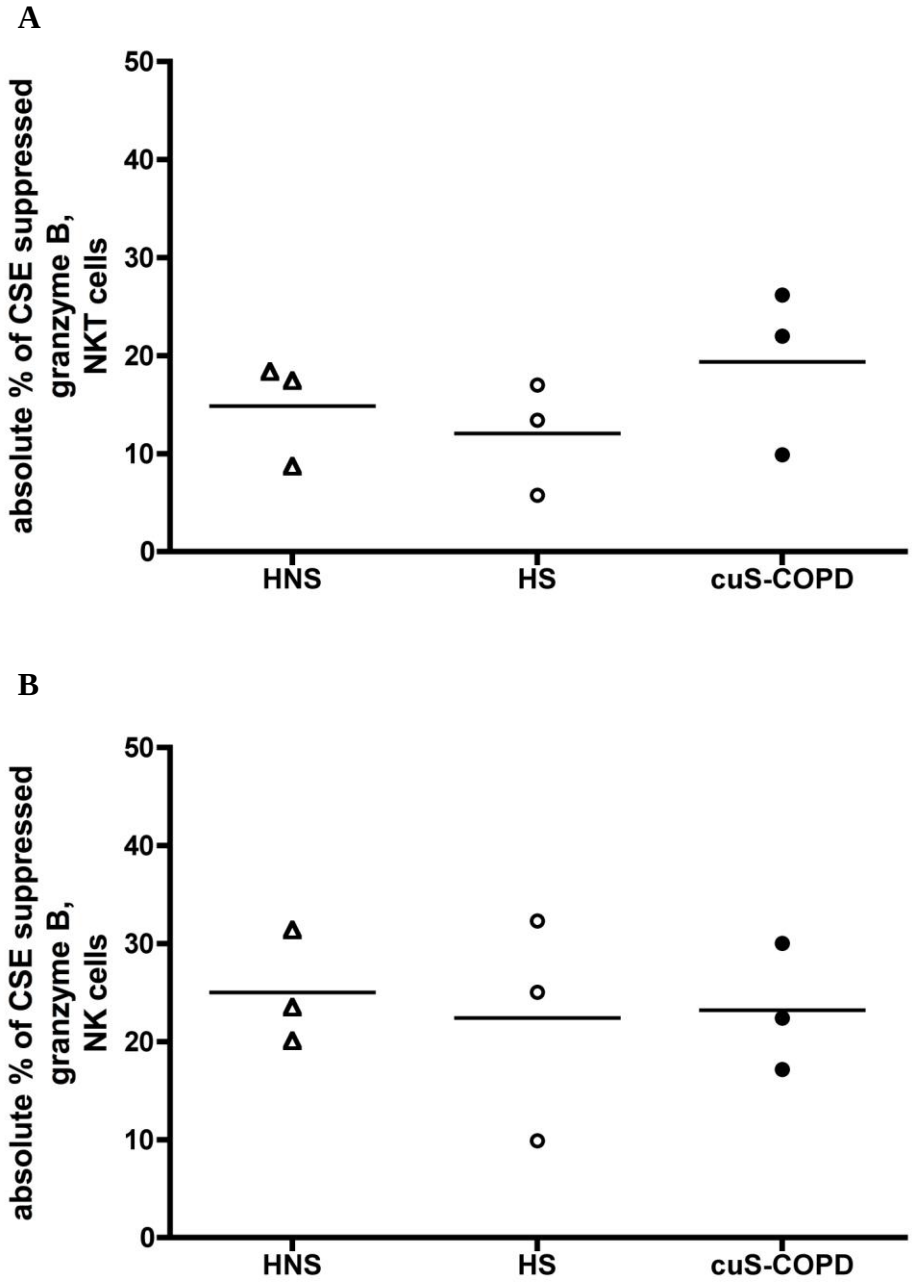



**Figure 3.3.9 CSE induced suppression on expression of granzyme B by IL-15 stimulated NKT-like cells and NK cells in three groups.** Absolute suppression levels were gained by using (% of IL-15 activated cells) minus (% of IL-15+CSE treated cells). All $p>0.05$. HNS (healthy non-smokers, n=3); HS (healthy smokers, n=3); cuS-COPD (current smokers with COPD, n=3).

IL-15 is a potent cytokine with the ability of stimulating many kinds of immune cells, such as memory $CD4^+$ T cells, naive or memory $CD8^+$ T cells, DCs, NKT cells and NK cells, which can further secrete more cytokines and chemokines to induce adaptive immune responses (Anderson et al., 1995, Cosman et al., 1995). Several studies demonstrated that cigarette smoke inhibited IL-12 and type-1 IFN production in activated PBMCs, which consequently decreased the IL-15 levels in healthy non-smokers (Mian et al., 2008, Mian et al., 2009). Also, both *in vitro* and *in vivo*, cigarette smoke significantly diminished IL-15 mRNA levels in poly I:C-activated PBMCs from healthy non-smoking participants. IL-15 production was markedly impaired in smoke-conditioned medium treated PBMCs upon poly I:C simulation. In result chapter 3.2, we showed that the CSE significantly reduced the activation levels of NKT-like cells from IL-15 treated PBMCs in healthy volunteers and ex-smokers with COPD. Also, CSE dramatically decreased the activation levels of NK cells in all groups, especially in COPD patients. Therefore, we examined whether the suppression induced by CSE on $CD56^+$ cells is directly correlated with IL-15 or through other immune mediators secreted by other cells. We found that CSE itself did not show any statistical impacts on the activation levels of $CD56^+$ cells compared to untreated cells. IL-15 highly activated NKT-like

cells and NK cells in both healthy volunteers and COPD patients. However, when CSE was added in the cultures, their activation levels were significantly reduced. This effect on NK cells was much more significant in current smokers with COPD. Interestingly, after 48 hour incubation, activation stages of NKT cells and NK cells showed different status (Figure 3.3.2). Most activated NKT cells are both CD69 and CD25 positive, whereas NK cells are mainly CD69 positive. This might be explained as followed. Cytokines released by NKT cells are always rapid and transient, with a peak of activity within 24 hours. After 24 hours, productions of these cytokines, for example IFN-$\gamma$, quickly return to baseline levels (Eberl and MacDonald, 1998). IFN-$\gamma$ is a good inducer for NK cell activation. This may explain why NKT cells were more quickly activated than NK cells.

Our group has illustrated that the cytotoxic functions of NK cells and NKT cells from PBMCs were both markedly reduced in COPD patients compared to healthy volunteers (Urbanowicz et al., 2009). *In vitro*, the percentage of perforin /granzyme B double positive NK cells was significantly lower in COPD patients compared to controls. Furthermore, Mian *et al* demonstrated that cigarette smoke inhibited IL-15 mediated NK cell cytotoxic killing ability with expressing lower NK cell

degranulation marker CD107a in healthy non-smokers (Mian et al., 2009). Granzymes, perforin and Fas-Fas ligand are three major killing molecules that facilitate NKT-like cells and NK cells performing their cytotoxic functions. To evaluate whether CSE induced suppression was linked to NK cell cytotoxicity, we analysed the expression levels of above three cytotoxic granules on IL-15 stimulated $CD56^{+}$ cells in the presence or in the absence of CSE. Surprisingly, CSE did not induce any remarkable impacts on expression levels of perforin and Fas-ligand in all groups. Perforin was highly expressed in all stimulating conditions whereas Fas-L showed very low levels. Of note, expression of granzyme B on IL-15 treated NKT-like cells was markedly reduced by CSE in healthy non-smokers. The remarkable effects of CSE on granzyme B expression by NK cells were also detectable in healthy non-smokers and current smokers with COPD. These findings were partially supported by other research. Morissette and colleague illustrated that no statistical differences were observed on the expression of perforin and granzyme B in peripheral NK cells from either healthy volunteers or smokers with evidence of emphysema. Protein expression of Fas-ligand was not detected by flow cytometry (Morissette et al., 2007). Although there was a markedly increase in soluble Fas in plasma from severe COPD patients, there were no differences

on its expression in mild or moderate COPD and healthy controls (Yasuda et al., 1998). Hodge *et al* also demonstrated that there were no significant changes in the expression levels of granzyme A by NK or NKT-like cells in the blood of COPD patients compared to controls (Hodge et al., 2013). Taken together, our data suggest that granzyme B, but not perforin and Fas-ligand, was a functional molecule attributing to the significant impairment NK cell killing ability in response to CSE in COPD patients.

KIRs are inhibitory receptors expressed by cytotoxic cells. We early found that, *in vivo*, expression levels of KIR (CD158e1) by killer cells including $CD8^+$ T cells and $CD56^+$ cells were negatively correlated with the activation levels of these cells in COPD patients. In this study, KIR expression by IL-15 incubated $CD56^+$ cells in response to CSE was analysed and compared between groups. Here, *in vitro*, we first time showed that there were no significant differences on KIR (CD158e1) expression by $CD56^+$ in different cell culture conditions. KIR expression was at very low levels in each group. Few studies were carried out to detect the KIRs expression in either healthy volunteers or COPD patients. However, one study reported that NKG2D expression was not inhibited in cigarette smoke treated PBMCs after poly I:C

induction (Mian et al., 2009). NKG2D is an activating receptor mainly expressed by killer cells and γ/δ T cells. We found, *ex vivo*, it was highly expressed in $CD56^+$ cells (averagely over 90%) in both healthy participants and COPD patients. Collectively, KIR (CD158e1) was not a functional inhibitory receptor for CSE induced suppression of $CD56^+$ cells activation in response to IL-15. Also, KIR levels from untreated cells *in vitro* (Figure 3.3.7) were lower than that *ex-vivo* (Fig 3.1.8). These might be due to, *in vitro*, cell interaction during 48 hour incubation. Activated cells might produce inflammatory molecules that could affect the functional receptor expression levels. However, more research should be carried out to clarify it.

Although CSE significantly suppressed $CD56^+$ cell activation and their granzyme B expression in IL-15 conditioned medium, there were no statistical differences between groups. This might be due to small sample size used in this study. We indeed found that the activation levels of NK cells inhibited by CSE in COPD patients were much more significant ($p<0.001$).

Several limitations should be considered in our study. Firstly, only small number of subjects in each group was recruited. Secondly, patient samples were only from current smokers

with COPD but not ex-smokers. Moreover, there are many cytotoxic granules such as granzyme A and granzyme K related to killing mechanisms of $CD56^+$ cells. However, proportions of NKT-like cells and NK cells are very low in PBMCs. We could just detect representative killing molecules (granzyme B, perforin and Fas-ligand) and one type of inhibitory receptors (KIR3DL, CD158e1) in our research due to cell numbers. The activation of $CD56^+$ cells is regulated by signal balance between inhibitory receptors and activating receptors. We could not present any significant differences about KIR expression on cells between groups (Figure 3.3.7). In future, other inhibitory or activating receptors, such as NKG2D and CD94, could be detected to find out whether they play potent roles in CSE suppressed activation.

In summary, this study showed for the first time that, CSE markedly suppressed IL-15 induced NKT-like cell activation and NK cell activation in healthy volunteers and current smokers with COPD. The granzyme B expression was also impaired on both cell types. Expression of perforin, Fas-ligand and KIR did not vary on $CD56^+$ cells either in response to IL-15 or co-stimulated CSE, in all groups. Our findings support the concept that cigarette smoke extract affects the cytotoxic functions of NKT-like and NK cell in COPD patients.

## 3.4 Cytotoxicity of $CD8^+CD56^-$ T lymphocytes in COPD

### 3.4.1 Introduction

Cytotoxic T lymphocytes (Tc cells, $CD3^+CD8^+CD56^-$) express the $\alpha\beta$ T cell receptor. The predominant function of Tc cells is to kill infected cells via producing cytokines, such as IFN-$\gamma$, and killing molecules like perforin and granzymes (Henkart, 1985, Zagury, 1982). Binding of Fas-ligand presented by Tc cells to Fas expressed by target cells can also induce apoptosis of infected cells (Nagata and Golstein, 1995). Abundant studies have shown abnormalities of Tc cells in COPD, such as in large airways sub-epithelium (O'Shaughnessy et al., 1997, Lams et al., 2000), peripheral airway (Saetta et al., 1998), parenchyma (Saetta et al., 1999), smooth muscle (Baraldo et al., 2004), bronchial arteries (Peinado et al., 1999, Saetta et al., 1999, Turato et al., 2002), peripheral blood (Urbanowicz et al., 2009), sputum samples (Urbanowicz et al., 2010), and small airways such as broncheoalveolar lavage (BAL) (Saetta et al., 2002, Saetta et al., 2003).

Cell-contact-dependent cytotoxicity is the hallmark of killer cell function (Russell and Ley, 2002). Cytotoxic T cells kill their

targets by programming them to undergo apoptosis. The elimination of infected cells without the destruction of healthy tissue requires the cytotoxic mechanisms of $CD8^+$ T cells to be both powerful and accurately targeted. Currently, abundant studies have investigated the cytotoxicity of killer cells, such as NK cells and $CD8^+$ T lymphocytes, from cancer patients (Jewett and Tseng, 2013, Levy et al., 2013, Maher and Davies, 2004). However, studies related to COPD are rare. Our group early demonstrated that cytotoxicities of NKT-like cells and NK cells were lower in PBMCs but higher in induced sputum from COPD patients compared to healthy volunteers (Urbanowicz et al., 2009, Urbanowicz et al., 2010). Chrysofakis *et al* also observed increased cytotoxic activity of Tc cells in induced sputum of COPD patients (Chrysofakis et al., 2004). In this chapter, we evaluated the cytotoxic activity of blood $CD8^+CD56^-$ T cells against the P815 cell line in four groups including healthy non-smokers, current healthy smokers, current smokers with COPD and ex-smokers with COPD.

### 3.4.2 Material and methods

#### 3.4.2.1 Study population and procedures

The criteria of participant enrolments are the same as previously introduced. There were thirteen participants

included in this study. Table 3.4.1 shows the demographic and spirometric data of the participants.

#### 3.4.2.2 PBMCs isolation

PBMCs were isolated from whole blood on a discontinuous Histopaque density gradient (Sigma). More details are provided in section 2.2.

#### 3.4.2.3 $CD8^+CD56^-$ T lymphocytes separation

Isolated PBMCs were incubated with anti-CD56 MicroBeads (Miltenyi Biotech Ltd, Surrey, UK) in MACS buffer containing sterile D-PBS (Sigma), 1% heat-inactivated fetal calf serum (FCS) (QB-Perbio) and 0.4% EDTA (Sigma) for 15 minutes at 4°C. $CD56^-$ cell fractions were obtained by cell suspension passing through a cold MS column attached to a magnet. Isolated $CD56^-$ fractions were washed and counted. To obtain $CD8^+CD56^-$ T lymphocytes, cells were incubated with Multisort Release Reagent for 10 minutes at 4°C, washed and then incubated with MultiSort Stop Reagent and $\alpha$-CD8 microbeads for 15 minutes at 4°C. Finally the labelled cells were separated on a refrigerated LS column. Detailed protocol for cell separation was shown on manufacturer's instructions. All isolated fractions were washed, counted and purity confirmed by flow cytometry (≥90%).

**Table 3.4.1 Demographic and spirometric valus of the CSE suppression study groups**

| | Healthy non-smokers | Current healthy smokers | cuS-COPD | exS-COPD |
|---|---|---|---|---|
| **Participants** | 4 | 4 | 3 | 2 |
| **Age (years)** | 55 （50-60） | 54 (51-60) | 55 (52-58) | 49 (46-52) |
| **Gender (M/F)** | 1/3 | 2/2 | 2/1 | 0/2 |
| **Packs/yrs** | 0 | 39 (33-42) | 43 (35-47) | 44 (39-49) |
| **Smoking status (Current/Ex)** | 0 | 4/0 | 3/0 | 0/2 |
| **Chronic bronchitis (Yes/No)** | 0 | 3/1 | 3/0 | 2/0 |
| **$FEV_1$ (% pred)** | 117 (102-128) | 103 (92-117) | 52 (46-60) | 57 (54-60) |
| **$FEV_1$/FVC (%)** | 97 (86-116) | 87 (66-106) | 49(45-53) | 47 (39-56) |
| **Δ$FEV_1$ post bronch** | 2.4 (1.7-2.9) | 1.8 (1.5-2.4) | 1.5 (1.2-1.6) | 1.4 (1.3-1.6) |
| **BMI ($kg/m^2$)** | 26.6 (22.6-28.3) | 25.2 (19.7-29.4) | 20.3 (15.8-25.1) | 20.1 (18.7-21.5) |
| **Inhaled GCS (on/off)** | N/A | N/A | 0/3 | 1/1 |
| **MRC dyspnoea scale** | N/A | N/A | 3 (2-4) | 3 (2-4) |
| **Distance walked in 6 min (m)** | N/A | N/A | 277 (196-312) | 302 (218-386) |
| **BODE Index** | N/A | N/A | 4 (3-6) | 5 (4-7) |

exS-COPD, ex-smokers with COPD; cuS-COPD, current smokers with COPD; $FEV_1$, forced expiratory volume in 1 second; pre, predicted value; FVC, forced vital capacity; BMI, body mass index; GCS, corticosteroid; MRC, medical research council; BODE, **B**MI, airflow **O**bstruction, **D**yspnoea and **E**xercise capacity.

### 3.4.2.4 Cell culture

For cytotoxicity assay, isolated $CD8^+CD56^-$ T cells were incubated for 24 hours at 37°C in 5% $CO_2$. Before cell incubation, the plate was coated with 2µg/ml anti-CD3 (in 50ul D-PBS solution each well) for a minimum of 2 hours at 37°C in 5% $CO_2$. Target cells (P815 cells) were grown in RPMI-1640 medium supplemented with 10% FCS at 37°C in 5% $CO_2$ and ready for use when they reached confluence.

### 3.4.2.5 $CD8^+CD56^-$ T lymphocytes Redirected Cytotoxicity Assay

The cytotoxicity of $CD8^+CD56^-$ T lymphocyte was measured with colorimetric quantification of lactate dehydrogenase LDH released from lysed target cells (cell line P815). More details are provided in section 2.7.

### 3.4.2.6 Flow cytometric analysis

Flow cytometric analysis of antibody labelled cells was performed using an EPICS Altra (Beckman Coulter, Luton, UK). More details are provided in section 2.9.

### 3.4.2.7 Statistical analysis

The statistical analysis was performed with Prism software, version 4.0c (GraphPad). Data were non-normally distributed

all are expressed as median and range, unless otherwise stated. Differences between the four groups of participants were tested using paramatric student's *t* test. Confidence interval 99% was selected to show two-tailed p values. P values of less than 0.01 were considered to indicate statistical significance.

### 3.4.3 Results

#### 3.4.3.1 Purity of Tc lymphocytes

The purity of isolated $CD8^+CD56^-$ T lymphocytes was determined by flow cytometry. Figure 3.4.1.A showed the representative dot plots of the proportion of $CD3^+CD8^+CD56^-$ T cells in each group, namely, healthy non-smokers (HNS, A i), current healthy smokers (HS, A ii), current smokers with COPD (cuS-COPD, A iii) and ex-smokers with COPD (exS-COPD, A iv). The purity of the immunomagnetically selected $CD3^+CD8^+$ T lymphocytes in isolated $CD8^+CD56^-$ fractions was over 90% (Figure 3.4.1.B).

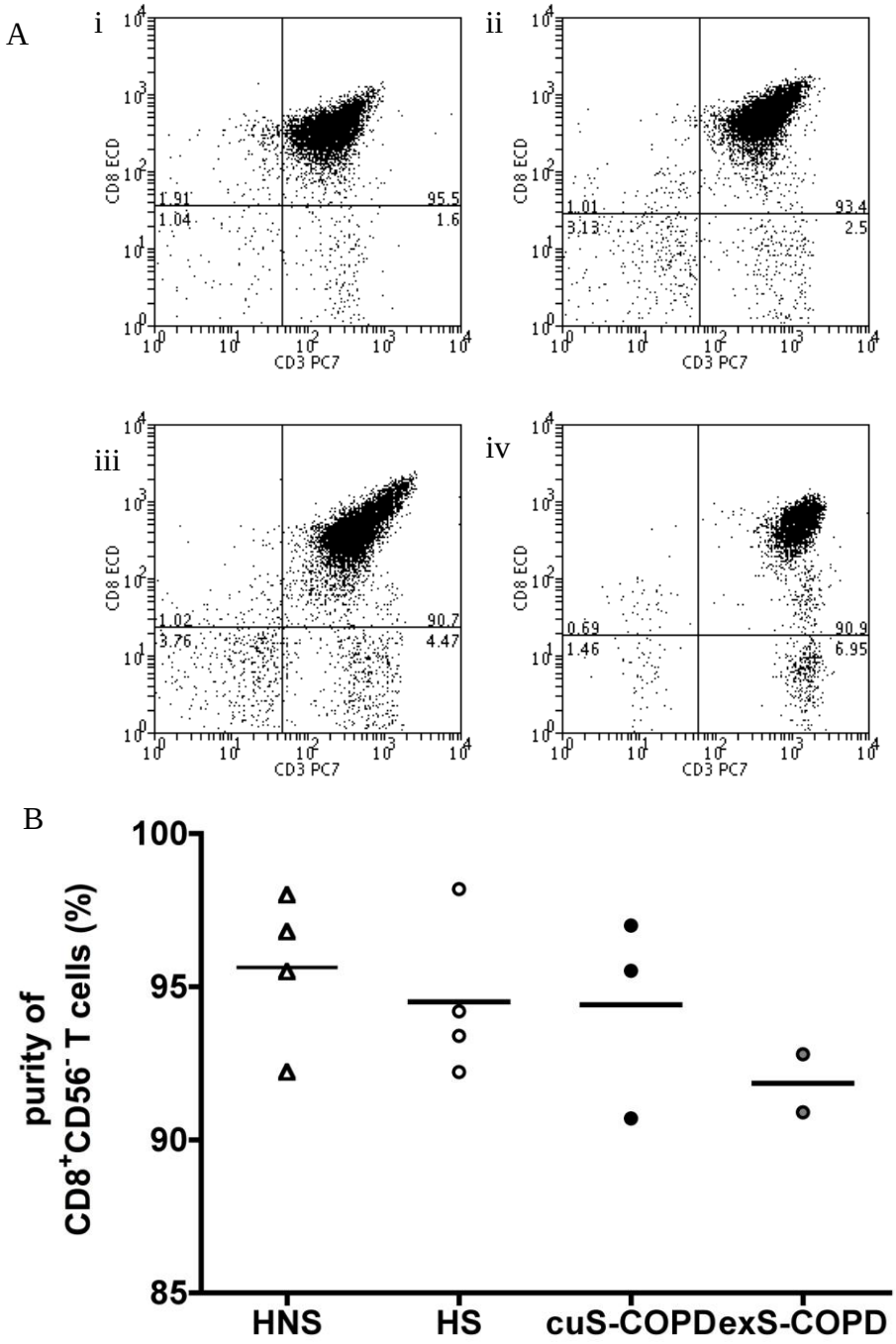



**Figure 3.4.1 Purity of immunomagnetically separated $CD8^+CD56^-$ T lymphocytes from the peripheral blood of the studied groups.** $CD8^+CD56^-$ T cells were isolated from PBMCs in four groups including health non-smokers (HNS, n=4), current healthy smokers (HS, n=4), current smokers with COPD (cuS-COPD, n=3), and ex-smokers with COPD (exS-COPD, n=2). (A) Representative dot plots of the purified $CD3^+CD8^+CD56^-$ T cells from PBMCs in each group, namely, i, NHS; ii, HS; iii, cuS-COPD; iv, exS-COPD. Percentage of purity was shown. (B) Purity of immunomagnetically separated $CD8^+CD56^-$ T lymphocytes in each group.

### 3.4.3.2 Cytotoxicity of $CD8^+CD56^-$ T lymphocytes in COPD

To test the cytotoxic activity of the immunomagnetically-purified $CD8^+CD56^-$ T cells, a non-radioactive colorimetric quantification of LDH released assay was performed (LDH is a stable cytosolic enzyme released upon cell lysis). *In vitro*, we compared the killing ability of $CD8^+CD56^-$ T cells against the P815 cell line at different ratios in the four participant groups. As shown in Figure 3.4.2, a dose dependent reduction was displayed on the cytotoxic ability of $CD8^+CD56^-$ T cells against P815 cells in each group. At ratio 40:1 (Figure 3.4.3.A), the cytotoxicity of $CD8^+CD56^-$ T cells was significantly less in both current smokers with COPD (21.6% ± 2.4%, $p<0.005$) and ex-smokers with COPD (27.57% ± 3.6%, $p<0.005$) compared to healthy non-smokers (63.47% ± 6.6%). The lysis ability of CD8+CD56- T cells were also dramatically decreased in current smokers with COPD (21.6% ± 2.4%, $p<0.005$) and ex-smokers with COPD (27.57% ±3.6%, $p<0.01$) compared to current healthy smokers (56.81% ± 9.7%) (Figure 3.4.3.A).

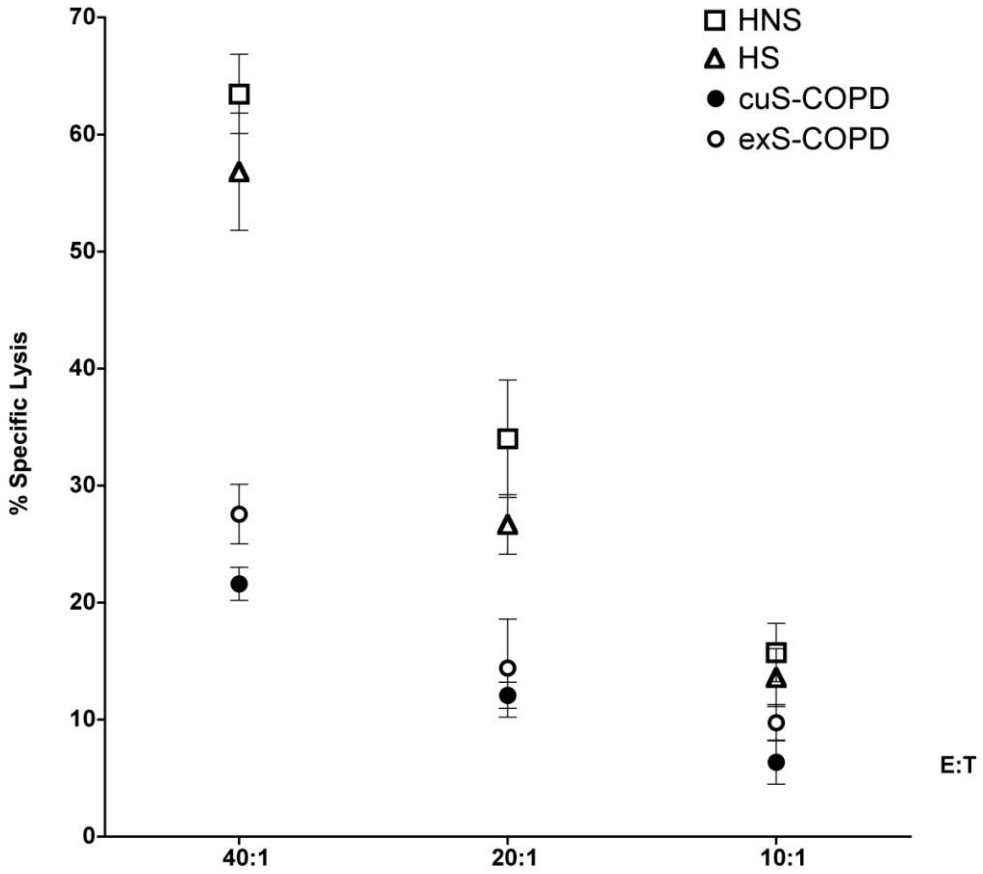



**Figure 3.4.2 Cytotoxic activity of $CD8^+CD56^-$ T cells towards P815 cells in studied groups.** $CD8^+CD56^-$ T cells were isolated from PBMCs of four interested groups, namely, health non-smokers (HNS, n=4), healthy smokers (HS, n=4), current smokers with COPD (cuS-COPD, n=3), and ex-smokers with COPD (exS-COPD, n=2). Purified $CD8^+CD56^-$ T cells were incubated with 2ug/ml of anti-CD3 for 24 hours before havesting. Cytotoxicity was assessed after 4 hours at the indicated effector to target (E:T) ratio. Data are mean ± standard deviation of different groups.

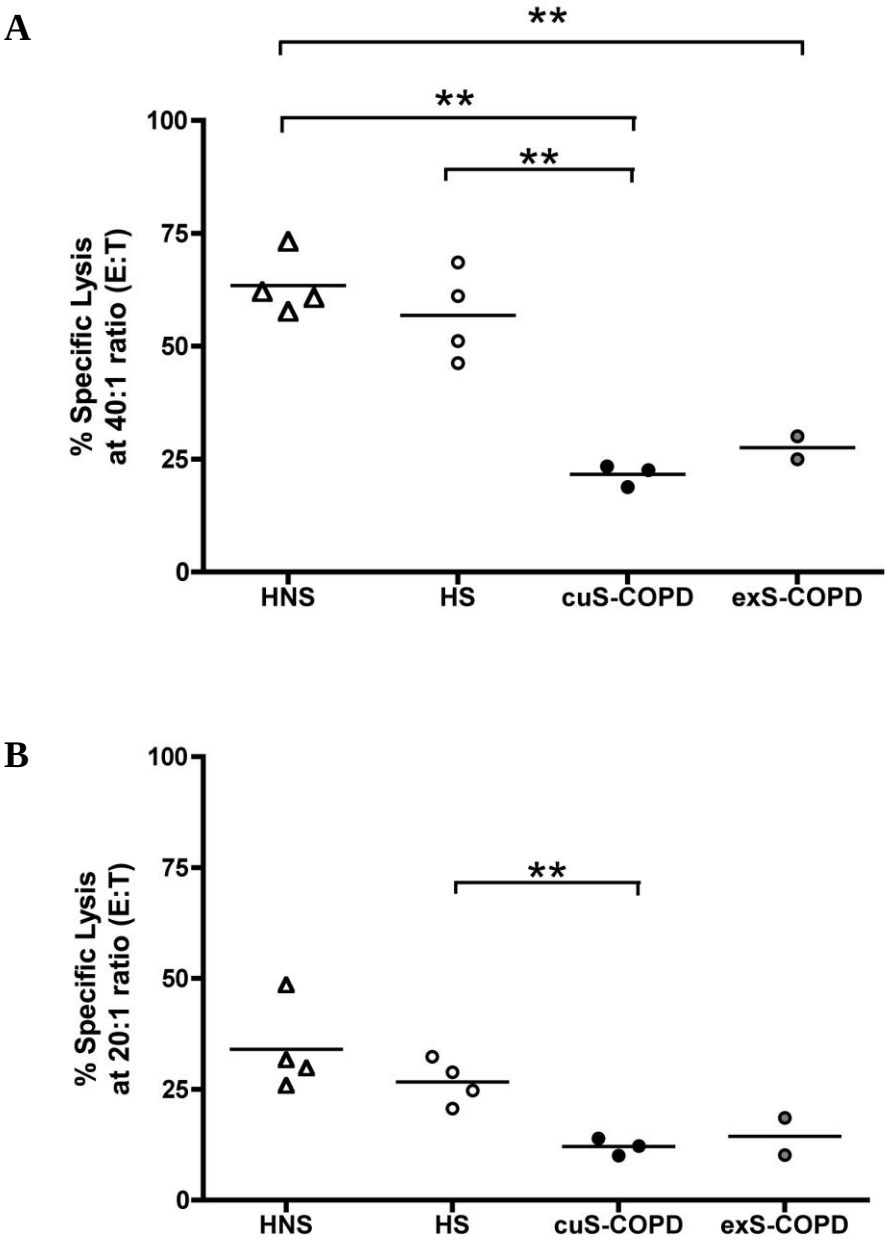



**Figure 3.4.3 Cytotoxic activity of $CD8^+CD56^-$ T cells against P815 cells at the effector to target ratio of 40:1 (panel A) and 20:1 (panel B).** Cytotoxicity detection for ratios 40:1 and 20:1 was performed in the same experiment in each sample. $CD8^+CD56^-$ T cells were immunomagnetically isolated from PBMCs of health non-smokers (HNS, n=4), current healthy smokers (HS, n=4), current smokers with COPD (cuS-COPD, n=3), and ex-smokers with COPD (exS-COPD, n=2). Significant differences were considered as p value<0.05 (*,p<0.01; **,p<0.005; ***,p<0.001).

Similar findings were also observed at ratio 20:1 (Figure 3.4.3.B). There were remarkable decreases on the cytotoxicity of $CD8^+CD56^-$ T cells from current smokers with COPD (12.1% ± 1.9%) compared to those from healthy non-smokers (34.0% ± 10.0%, $p<0.01$) and current healthy smokers (26.7% ± 5.1%, $p<0.005$). There were no significant differences on the lysis ability of Tc cells between healthy non-smokers and current healthy smokers at each ratio.

### 3.4.4 Discussion

Here we demonstrated that $CD8^+CD56^-$ T lymphocytes from PBMCs showed significantly less cytotoxic activity against P815 cells in COPD patents compared to healthy non-smokers and healthy smokers. Also, dose dependent reductions were exhibited on the cytotoxicity of $CD8^+CD56^-$ T cells in each group. These findings were in accordance with an earlier study that showed the cytotoxicity of NK cells and NKT-like cells from PMBCs was significantly lower in COPD patients in contrast to healthy volunteers (Urbanowicz et al., 2009).

Target-cell death induced by $CD8^+CD56^-$ T cells can be initiated by various signals (Plataki et al., 2006). For example, when cytotoxic T cells are mixed with target cells and rapidly brought into contact by centrifugation, they can program

antigen-specific target cells to die by secretion of preformed effector molecules and cytokines, such as perforin, granzymes and INF-$\gamma$. K562 cell line was previously selected as the target cells in this research. However, no remarkable findings were observed between COPD patients and healthly groups. Previous studies have shown that K562 cells do not express HLA-A, B, C, or HLA-DR on their surface that are recognized by $CD8^+$ T cells to induce cell cytotoxicity (Garson et al., 1985, Maziarz et al., 1990). Here we used P815 cell line as target cells. The lysis ability of $CD8^+$ T cells against P815 cells was significantly reduced in COPD groups compared to healthy volunteers. This was supported by other studies that showed $CD56^-$ T cells, which were NKG2D-positive, were not cytotoxic against K562 cells (Karimi et al., 2005, Trinchieri, 2003, Verneris et al., 2004). NKG2D might account for this reason. Besides MHC class I molecule dependant pathway, cytotoxic T cells also induce activating signal by MHC-unrestricted and TCR-independent pathway (Schmidt-Wolf et al., 1993). NKG2D is a representative MHC-unrestricted activation receptor expressed by human NK cells, $CD8^+$ T cells and $\gamma\delta$ T cells. Multiple studies illustrate that NKG2D ligation leads to different functional outcomes on killer cells (Diefenbach et al., 2000, Groh et al., 2001, Jamieson et al., 2002, Pende et al., 2001). Groh *et al* showed that NKG2D augmented cytotoxicity

of $CD8^+$ T cells only when cytotoxic T cells and target cells were MHC matched (Groh et al., 2001). Another research also confirmed that NKG2D-blocking antibodies dramatically attenuated the cytolysis of $CD8^+$ T cells in tumor targets (Verneris et al., 2004).

DAP10 and DAP12 are two well-identified adapter proteins associated with NKG2D in human cytotoxic cells (Rosen et al., 2004). Studies confirm that NKG2D, together with the DAP10 and DAP12 molecules, has been shown to play a unique role in the mechanism of cytotoxicity of T cells (Karimi et al., 2005, Trinchieri, 2003, Verneris et al., 2004). Double knockdown of DAP10 and DAP12 reduced much of the killing ability of activated $CD8^+$ T cells in PBMCs from healthy volunteers (Kakimi et al., 2000). However, DAP10 and DAP12 were associated with not only NKG2D receptor but also other receptors such as KIRs and CD94-NKG2C receptors (Lanier, 2009). DAP12-associated KIR has been indentified on the subsets of human effector T cells in mice (Feng et al., 2005). Defining the DAP12-related KIRs in human are beyond our study, however, in future, the relationship between KIRs expression and cytotoxicity of killer cells may be another prospective study in COPD. Indeed, in an earler section we showed that *ex vivo* expression of KIRs, but not NKG2D, on

$CD8^+$ T cells were highly reduced in current smokers with COPD (Figure 3.1.8).

The relatively low participant numbers, especially the number of ex-smokers with COPD, should be pointed out. We did not find out any differences on the cytotoxic ability of $CD8^+CD56^-$ T cells between current smokers with COPD and ex-smokers with COPD. However, the significant differences between healthy and patient groups could partially explain the finding that the cytotoxicity of $CD8^+$ $CD56^-$ T cells was disease-related rather than smoking *per se*.

In summary, this study has shown that the cytotoxicity of $CD8^+CD56^-$ T cells was significantly reduced in the peripheral blood of COPD subjects (both current and ex) compared to healthy non-smokers and healthy current smokers.

## 3.5 Killer cell activation in bronchoalveolar lavage fluid from COPD

### 3.5.1 Introduction

In the last decade, there have been many studies performed on bronchial biopsies, lung parenchyma and peripheral blood obtained from COPD patients compared with healthy non-smokers and smokers with normal lung function. Bronchoscopy with bronchoalveolar lavage (BAL) is an important research tool for assessing airway inflammation in a variety of inflammatory lung diseases. Increased numbers of neutrophils (Battaglia et al., 2007), $CD1a^+$ immature DCs (Casolaro et al., 1988, Soler et al., 1989), macrophages (Barnes, 2004d, Saetta et al., 1993, Saetta et al., 1997, Wallace et al., 1992), eosinophils (Saetta et al., 1996, Saetta et al., 1994), $CD8^+$ T cells (Saetta et al., 2002, Saetta et al., 2003), $\gamma/\delta$ T cells (Ekberg-Jansson et al., 2000, Pons et al., 2005) and regulatory T cells (Smyth et al., 2007) were detected in the BAL fluid from either current healthy smokers or current smokers with COPD. However, the number of iNKT cells from BAL was significantly decreased (less than 2% of total $CD3^+$ T cells) in subjects with stable or exacerbated COPD (Vijayanand et al., 2007). Besides immune cells,

abnormal levels of immune molecules were also detected in BAL from COPD patients. The concentration of CXCL1 (Traves et al., 2002), CXCL5 (Tanino et al., 2002), CCL2 (Capelli et al., 1999, de Boer et al., 2000, Traves et al., 2002), GM-CSF (Balbi et al., 1997), ET-1 (Bacakoglu et al., 2003, Chalmers et al., 1999), histamine (Kalenderian et al., 1988) and tryptase (Bessa et al., 2008, Kalenderian et al., 1988) were all significantly increased in BAL fluid from COPD patients. Levels of both IL-4 released by $CD8^+$ T cells and IFN-$\gamma$ released by $CD4^+$ T cells were also elevated in the BAL fluid from COPD patients (Barczyk et al., 2006). Increased xanthine/xanthine oxidase activity has been observed in BAL fluid from COPD patients (Pinamonti et al., 1998). The expression of perforin and granzyme B on $CD8^+$ T cells from BAL fluid were also significantly increased in COPD patients and healthy smokers compared non-smokers (Hodge et al., 2006).

To date, the study on the killer cell activation from BAL in COPD is rare. In this chapter, we aim to examine the activation levels of killer cells including $CD8^+$ T cells, NKT-like cells and NK cells from BAL in COPD patients.

### 3.5.2 Material and Methods

#### 3.5.2.1 Study population and procedures

The criteria of participant enrolments are the same as previously introduced. There were only four current smokers with COP included in this study. Table 3.5.1 shows the demographic and spirometric data of the participants.

#### 3.5.2.2 BAL collection

Bronchoscopy with BAL was performed by a clinician from the Queen's Medical Centre. More details are provided in Section 2.8.

**Table 3.5.1 Demographic and spirometric valus of the BAL study**

| Current smokers with COPD | Age (years) | Gender (M/F) | Packs/ year | Chronic bronchitis (Yes/ No) |
|---|---|---|---|---|
| **n=4** | 49 (43-57) | 2/2 | 41 (38-46) | 4/0 |

| FEV1 (% pred) | $FEV_1$/ FVC (%) | $\Delta FEV_1$ post bronch | BMI ($kg/m^2$) |
|---|---|---|---|
| 61 (50-72) | 37 (31-44) | 1.6 (1.4-1.9) | 19.7 (16.6-22.5) |

### 3.5.2.3 BAL cell collection

Before centrifugation of BAL fluid, two 50ml centrifuge tubes were weighted on an electronic scale. Samples were pipetted into the tubes and volumes of them were noted. Filled tubes were reweighted and the weight of recovered BAL fluid was calculated. More details are provided in Section 2.8.

### 3.5.2.4 Extracellular staining

To determine the cell populations in the BAL, extracellular staining for these cells were performed as described previously. Cells were labeled with fluorescently conjugated antibodies including CD3 (PC7), CD8 (ECD) and CD56 (PE) to identify the proportion of cell population of interest. CD25 (FITC) and CD69 (PC5) were also added to detect the activation of interested cell types. All samples were analysed by flow cytomentry.

### 3.5.2.5 Flow cytometric analysis

Flow cytometric analysis of antibody labelled cells was performed using an EPICS Altra (Beckman Coulter, Luton, UK). More details are provided in Section 2.9.

### 3.5.2.6 Statistical analysis

The statistical analysis was performed with Prism software, version 4.0c (GraphPad). Differences between four cell types including $CD8^+$ T cells, $CD4^+$ T cells, NKT cells and NK cells from COPD smokers were tested using the parametric student's *t* test. Confidence interval 99% was used to show two-tailed p values. P values of less than 0.01 were considered to indicate statistical significance.

### 3.5.3 Results

*Killer cell activation in BAL from COPD patients ex vivo*

All individuals had similar total mononuclear cell numbers, which were within the normal range (data not shown). Representative flow analysis dot plots showed Forward Scatter (FS) vs Side Scatter (SS) for identification of live and dead cells (Figure 3.5.1.A), along with dot plots for identification of $CD8^+$ T-lymphocytes ($CD3^+CD8^+$), $CD4^+$ T-lymphocytes ($CD3^+CD4^+$), NKT-like cells ($CD3^+CD56^+$), and NK cells ($CD3^-CD56^+$) (Figure 3.5.1.B). In addition dot plots for CD69 and CD25 staining of these four cell types from BAL of a COPD patient ar presented in Figure 3.5.2. Cells are designated activated when they express one or both of the activation markers (i.e., CD69 positive, CD69/CD25 double positive or CD25 positive). $CD8^+$ T cells (92.0%, 89.1-97.1; $p<0.001$),

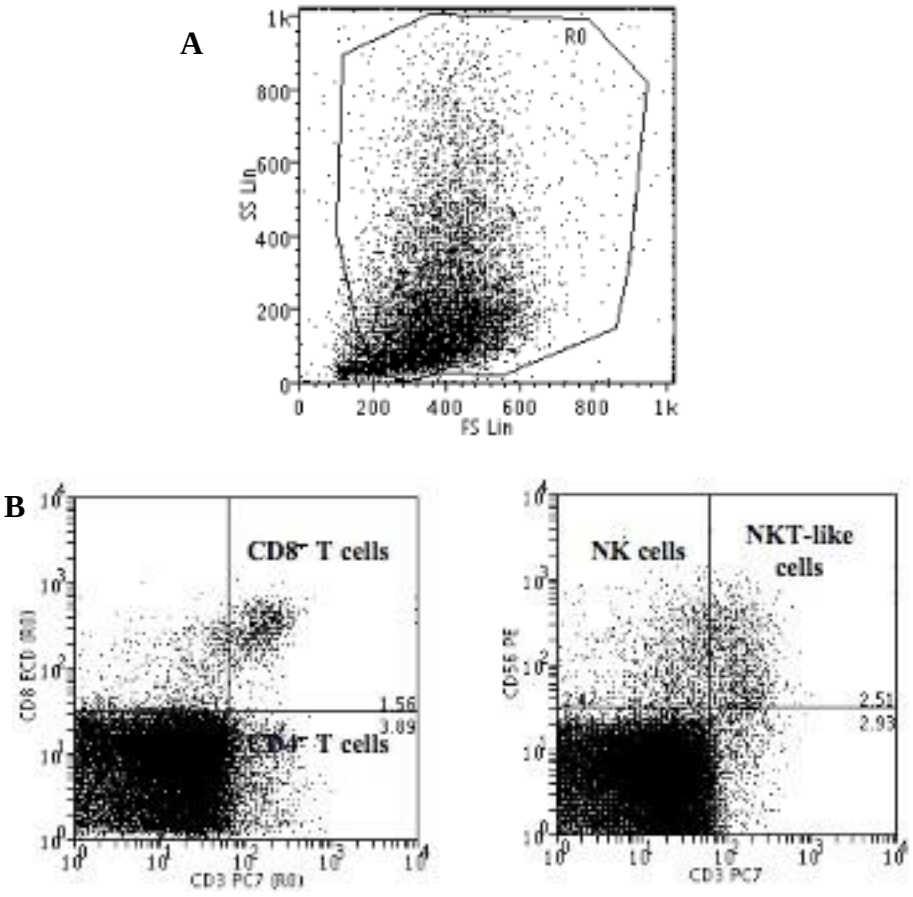



**Figure 3.5.1 Representative dot plots gating for live/dead cells and identification cell populations from current smokers with COPD.** (A) Forward Scatter (FS) vs Side Scatter (SS) plot for indentification of live and dead cells; (B) Indentification of $CD8^+$ T cells ($CD3^+CD8^+$), $CD4^+$ T cells ($CD3^+CD4^+$), NKT-like cells ($CD3^+CD56^+$) and NK cells ($CD3^-CD56^+$).

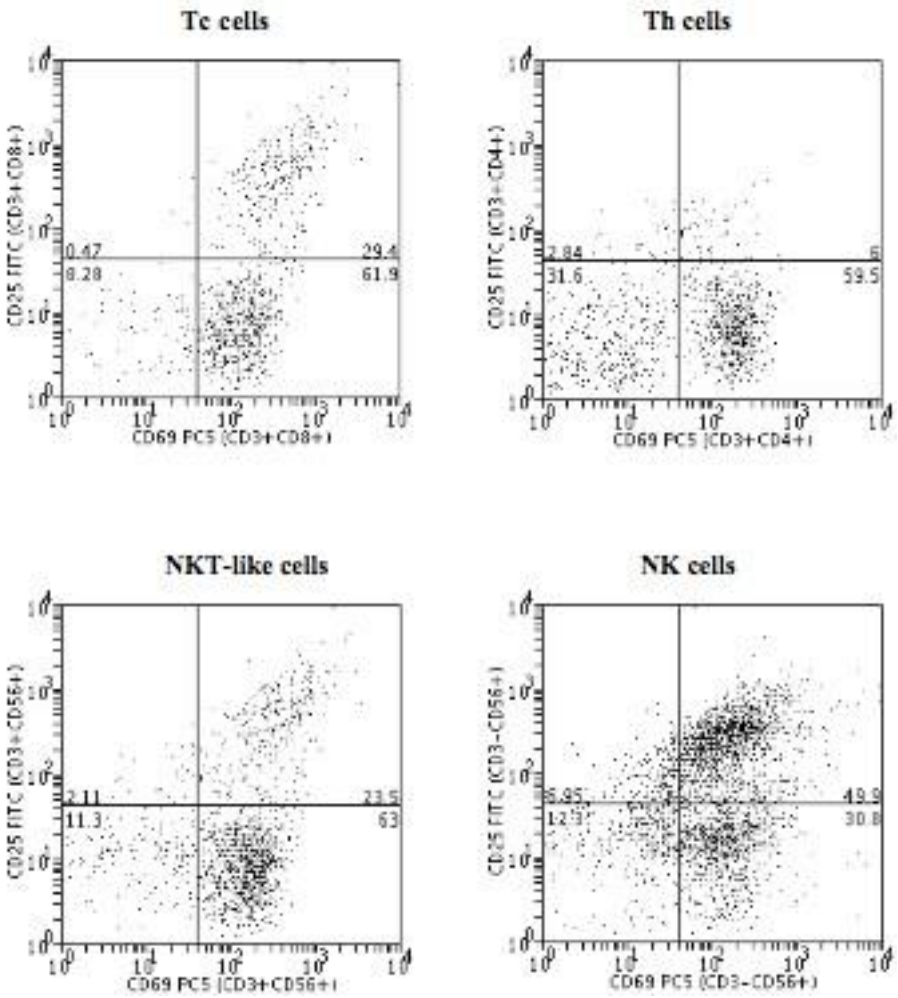



**Figure 3.5.2 Representative dot plots for identification of activation of cells of interst from a current smoker with COPD.** Activation is designated by expressing either CD69 alone (lower right quadrant), CD69 and CD25 (upper right quadrant) or CD25 alone (upper left quadrant).

NKT-like cells (96.5%, 87.7-99.2; p<0.001) and NK cells (94.5%, 88.7-97.3; p<0.001) were all highly and significantly activated in compared to $CD4^+$ T cells (46.9%, 40.2-68.5) in COPD patients (Figure 3.5.3). CD69 and CD25 represent early and late activation markers for cell activation respectively. As shown in Figure 3.5.4, activated $CD8^+$ T cells and NK cells primarily express CD69, whereas the major proportion of activated NKT-like cells is CD69 and CD25 double positive. All cells analysed expressed low level of CD25.

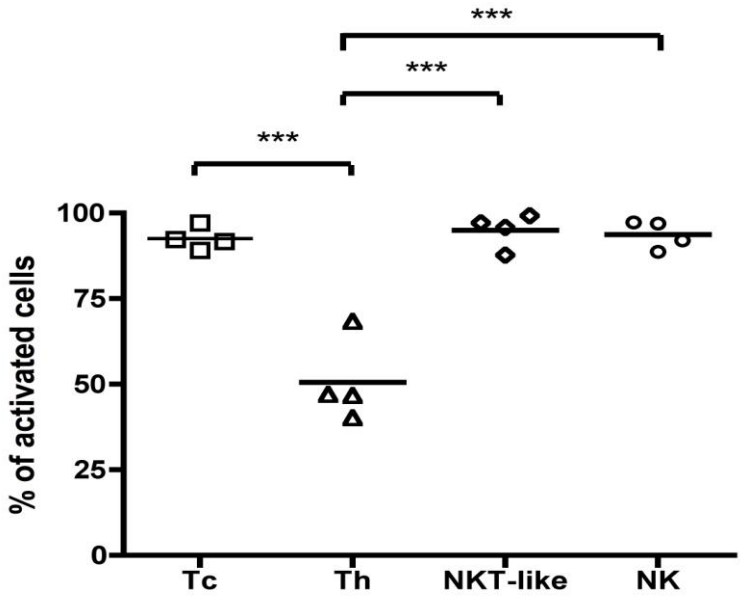



**Figure 3.5.3 Activation of cells of interest including $CD8^+$ T cells (Tc), $CD4^+$ T cells (Th), NKT-like cells and NK cells from BAL fluid of current smokers with COPD (n=4).** Tc, NKT-like and NK cells were all significantly activated compared Th cells from BAL of current smokers with COPD (p<0.001).

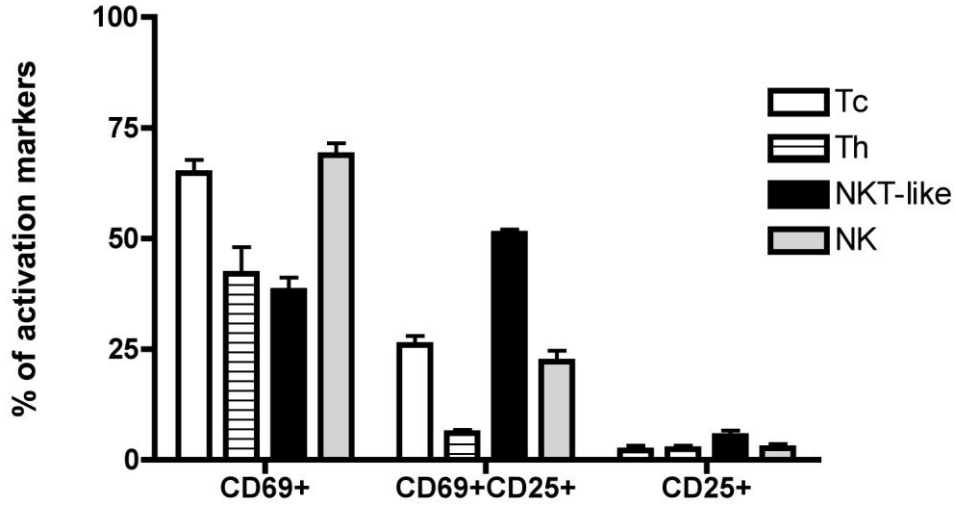



**Figure 3.5.4 Activation stages of cells of interest including CD8$^+$ T cells (Tc), CD4$^+$ T cells (Th), NKT-like cells and NK cells from BAL fluid of current smokers with COPD (n=4).** Tc, NKT-like and NK cells were all significantly activated compared Th cells from BAL of current smokers with COPD.

### 3.5.4 Discussion

Bronchoscopy with BAL is an important research tool for assessing airway inflammation in a variety of inflammatory lung diseases. Many studies have shown the abnormal numbers of immune cells and molecules from BAL in COPD. Here we showed that killer cells including CD8$^+$ T cells, NKT-like cells and NK cells were all highly activated compared to CD4$^+$ T cells from BAL of current smokers with COPD. This finding was supported by other studies. CD8$^+$ T-lymphocytes from BAL fluid of smokers with or without COPD were significantly activated compared to healthy non-smoking

volunteers (Roos-Engstrand et al., 2009). Also, increased proportion of $CD8^+$ T cells expressing CD69 was observed in the lung tissue of COPD patients compared to non-smokers (Freeman et al., 2010). However, in our study we could not conclude that high levels of killer cell activation were disease specific due to lack of healthy controls.

CD69 and CD25 represent early and late activation markers respectively for cell activation. Interestingly, we found that the major proportion of activated NKT-like cells was CD69 and CD25 double positive in BAL of current smokers with COPD, whereas $CD8^+$ T cells and NK cells mainly express CD69 (early activation marker) *ex vivo*. This finding was partially supported by Freeman *et al* study. They found increased proportion of $CD8^+$ T cells expressing CD69 in the lung tissue of COPD patients compared to non-smokers (Freeman et al., 2010). NKT cells, a subset of T lymphocytes but also express NK cell surface markers, exhibit both pro-inflammatory and immuregulatory characteristics. Cytokines released by NKT cells are always rapid and transient, with a peak of activity within 24 hours. After 24 hours, productions of these cytokines, for example IFN-$\gamma$, quickly return to baseline levels (Eberl and MacDonald, 1998). This may explain why NKT cells were more quickly activated than $CD8^+$ T cells and NK cells.

Here we also showed the activation level of $CD4^+$ T cells was lower compared to interested killer cells in COPD patients. This is consistant with our findings in PBMCs and sputum that both showed low level activation of Th cells in both healthy groups and COPD subjects. However, there were no control groups in BAL study. In future, we could compare the expression of avtivatioin markers of CD69 and CD25 between healthy volunteers and patient groups. Also, to identify whether those $CD4^+$ T cells were regulatory T cells, FoxP3 staining could be applied.

Overactivation of killer cells can induce hypersecretion of inflammatory cytokines and chemokines that consequently cause other types of cell activation, and finally contribute to the lung tissue damage in the COPD patients. Although here we could not conclude these highly activated cells were disease related because of no control samples, in future, it may be worth investigating the killer cells activation and their functional receptor expression in BAL fluid from both healthy volunteers and COPD patients, which provide new insights on the role of killer cells and contributing to a better knowledge of the pathogenesis of COPD.

# Discussion

COPD is characterised by poorly reversible airflow obstruction and airway inflammation with high morbidity and mortality (Celli and MacNee, 2004). As an inflammatory disease, both innate and adaptive immune system show abnormal features in COPD (O'Shaughnessy et al., 1997, Saetta et al., 1998, Baraldo et al., 2004, Barnes, 2004b, Demedts et al., 2007, Urbanowicz et al., 2009) that eventually lead to chronic obstructive bronchiolitis, emphysema and mucus hypersecretion.

Activated neutrophils (Damiano et al., 1986, Galdston et al., 1977), macropahges (Molet et al., 2005, Montano et al., 2004), DCs (Demedts et al., 2007) and $CD8^+$ T lymphocytes (Roos-Engstrand et al., 2009) have been reported in airways or sputum of COPD patients compared to normal non-smokers. However, studies on the activation levels of killer cells including $CD8^+$ T cells, NKT-like cells and NK cells from PBMCs are unexplored. Previous studies also demonstrated the potential involvement of the three main classes of human killer cells, namely, $CD8^+$ T-lymphocytes, NKT-like cells and NK cells in COPD (Paats et al., 2012, Roos-Engstrand et al., 2009, Roos-Engstrand et al., 2010, Urbanowicz et al., 2009) that play key roles in inflammatory responses.

Here we showed, *ex vivo*, for the first time that activation phenotype of these cells in the peripheral blood. Activation levels $CD8^+$ T-lymphocytes, NKT-like cells and NK cells were significantly higher in current smokers with or without COPD in comparison with healthy non-smokers and ex-smokers with COPD. This finding indicated a systemic influence of smoking on killer cell activation, irrespective of the presence of airflow limitation. We also found that NK cell activation was positively correlated with the number of cigarettes currently smoked in COPD patients. No correlations between cell activation and lung function ($FEV_1$ percentage of predicted) were found. These data indicate that in peripheral blood, cell activation levels are more related to current smoking habit rather than disease. A previous study examining the effect of smoking cessation has shown that $CD8^+$ T-lymphocytes from BAL fluid of smokers with or without COPD expressed high levels of CD69 and CD25 activation markers (Roos-Engstrand et al., 2009). Also, an increased proportion of $CD8^+$ T cells expressing CD69 was observed in the lung tissue of COPD patients compared to non-smokers (Freeman et al., 2010). Sputum T-lymphocytes have been shown to express higher levels of CD103 and CD69 than blood lymphocytes suggesting higher numbers of activated intraepithelial phenotype T-lymphocytes in the lung of COPD patients (Leckie et al., 2003).

Also, in our study, $CD4^+$ T cells expressed very low levels of activation markers, namely, CD69 and CD25, in all groups. CD25 is also a cell marker for regulatory T cells. Therefore, we hypothesised those $CD4^+CD25^+$ T cells might mainly be regulatory cells rather than activated cells. To examine this further we generated Forward Scatter (FS) vs Side Scatter (SS) plots that showed, unlike other activated cell types expressing CD25 that were larger and more granular, those $CD4^+CD25^+$ T cells were primarily small and less granular and thus quite probably inactivated lymphocytes. However, to confirm this observation further studies are required, such as staining for FoxP3. Although two studies have found highly activated Th1 cells in the lung of ex-smokers with COPD and in the smalll airway wall of smokers with severe COPD by detecting their chemokine and cytokine secretion, those samples are actually from COPD patients with emphysema or co-suffering from cancer (Turato et al., 2002).

KIR (CD158e1) expression by killer cells was significantly lower in peripheral blood of current smokers with or without COPD compared to healthy non-smokers. This finding was opposite to the activation levels of these killer cells. Signalling balance between activating receptors and inhibitory receptors is one of the crucial factors determining cell activation. Similar

finding was shown in Hodge *et al* study. CD94 is another inhibitory receptor expressed by NKT-like cells and NK cells (Lazetic et al., 1996). Decreased expression of CD94 on both NKT-like cells and NK cells were detected in PBMCs from COPD patients compared to healthy volunteers (Hodge et al., 2012). However, they did not detect any significant differences on the cell activation levels of NKT-like and NK cells (only CD69 expression) between groups. Therefore, our findings provided the first demonstration of association of cell activation and KIR expression.

We also showed that activating receptor NKG2D was highly expressed by $CD8^+$ T cells, NKT-like cells and NK cells but not $CD4^+$ T cells, which was in accordance with previous studies that demonstrate NKG2D receptors are constitutively expressed and almost exclusively on cytotoxic cells including $CD8^+$ T cells, NKT cells, NK cells and T cells (Maasho et al., 2005). There were no significant differences of NKG2D expression on $CD8^+$ T cells, $CD4^+$ T cells, NKT-like cells and NK cells respectively, in the four groups. This further supports the proposal of increased peripheral blood killer cell activity in smokers due to the increased ratio of activating to inhibitory receptors.

Of note, our group has published data showing, *ex vivo*, the activation levels of killer cells in sputum samples from COPD patients are elevated (Wang et al., 2013). Unlike systemic activation, lung activation is due specifically to COPD, irrespective of current smoking status.

In this study, activation levels of cells from BAL of COPD patients were examined. $CD8^+$ T cells, NKT-like cells and NK cells were all highly activated compared to $CD4^+$ T cells in BAL fuild from COPD patients *ex vivo*. This finding was similar to those we observed in PBMCs from current smokers with COPD. Moreover, $CD8^+$ T cells and NK cells showed early activation stage (mainly expressing CD69), whereas the main proportion of activated NKT-like cells were CD69 and CD25 double positive. However, it was hard to conclude whether it was COPD specific due to no control samples from healthy participants. This is because the procedure of taking BAL samples is indeed very uncomfortable for those volunteers.

Cytotoxicity of $CD8^+$ T cells was also investigated in our study. We demonstrated that $CD8^+CD56^-$ T lymphocytes from PBMCs showed significantly less cytotoxic activity against P815 cells in COPD patents compared to healthy non-smokers and healthy smokers. Also, dose dependent reductions were

exhibited on the cytotoxicity of $CD8^+CD56^-$ T cells in each group. This finding was in accordance with previous research on lysis ability of $CD56^+$ cells. The cytotoxicity of $CD56^+$ cells, including NK cells and NKT-like cells, from PMBCs against K562 cell line was significantly lower in COPD patients in contrast to healthy volunteers (Urbanowicz et al., 2009).

Of note, K562 cell line was previously selected as target cells in this research. However, no remarkable findings were observed between COPD patients and health groups. Here we used P815 cell line as target cells. Interestingly, the lysis ability of $CD8^+$ T cells against P815 cells was significantly reduced in COPD groups including both current and ex-smokers with COPD compared to healthy volunteers. Anti-CD3 monoclonal antibody induces cytolysis by cross-linking $CD3^+$ effector and IgG-$FcR^+$ target cells. After coating with P815 cells, anti-CD3 can induce P815 cells bearing the relevant IgG-FcR and further perform as antigen presenting cells to activate CD8+ T cells (van de Griend et al., 1987). Furthermore, other studies that showed NKG2D-positive $CD56^-$ T cells were not cytotoxic against K562 cells (Karimi et al., 2005, Trinchieri, 2003, Verneris et al., 2004). Besides MHC class I molecule dependant pathway, cytotoxic T cells also induce activating signal by MHC-unrestricted and TCR-independent pathway

(Schmidt-Wolf et al., 1993). Groh *et al* showed that NKG2D augmented cytotoxicity of $CD8^+$ T cells only when cytotoxic T cells and target cells were MHC matched (Groh et al., 2001). Another research also confirmed that NKG2D-blocking antibodies dramatically attenuated the cytolysis of $CD8^+$ T cells in tumor targets (Verneris et al., 2004).

We did not find any differences on the cytotoxic ability of $CD8^+CD56^-$ T cells between current smokers with COPD and ex-smokers with COPD. However, the significant differences between healthy and patient groups could partially reveal the finding that the cytotoxic of $CD8^+$ $CD56^-$ T cells was disease-related rather than smoking *per se*.

Cigarette smoking, as the major risk factor in the development of COPD, affects structural and functional changes in the respiratory ciliary epithelium (Cantin et al., 2006, Mio et al., 1997, Wyatt et al., 1999), lung surfactant protein (Honda et al., 1996, Subramaniam et al., 1996) and immune cells (Mohan, 2002a).

Here we investigate the impact of cigarette smoke extract (CSE) on $\alpha$-CD3, IL-2 or IL-15 activated killer cells including Tc cells, NKT-like cells and NK cells, and further compared the

absolute suppression levels induced by CSE on those cells upon different activation between four groups, namely, healthy non-smokers, current healthy smokers, current smokers with COPD and ex-smokers with COPD.

Optimal concentration of each activator and incubation time for cell culture was achieved by a preliminary protocol. Tc cells, NKT-like cells and NK cells were all highly activated when the concentration of $\alpha$-CD3 increased to 1ug/ml. However, in preliminary experiments, dose-dependent increases in the activation levels of NK cells were also observed, although NK cells do no express CD3/TCR complex on their cell surface. To our knowledge, this could be easily explained. $\alpha$-CD3 has the capacity of activating $CD4^+$ T cells, Tc cells and NKT cells that further release immune mediators such as IL-2 or IL-15 (Smyth et al., 2005, Thompson et al., 1989). Furthermore, CSE also has effects on DCs and macrophages that also secret high amount of IL-15 (Carson et al., 1995, Jonuleit et al., 1997). Both IL-2 and IL-15 are good sources for NK cell activation (Waldmann, 2006).

In our study, activation levels of killer cells were not significantly different in the absence or presence of CSE at increasing dilutions in healthy volunteers, without other

stimulations. These findings were in agreement with other groups who detected the CSE effects on DCs or NK cells from healthy volunteers (Mian et al., 2009, Mortaz et al., 2009). According to previous studies and our detection, we selected 1% of CSE for further experiments because it did not cause any toxic effects as revealed by MMT assay (Mian et al., 2008, Mian et al., 2009, Stringer et al., 2007).

The effects of CSE on killer cells were explored not only in healthy volunteers but also COPD patients in our study. Three significant results should be highlighted. Firstly, CSE markedly suppressed activation levels of IL-15 treated cells for all cell types in all groups. Secondly, in contrast to Tc cells and NKT cells, NK cells were the only cell type which activation levels upon different stimulation were all significantly decreased when CSE was added in all groups. Thirdly, current smokers with COPD were the only group that showed CSE had statistically significant effects on all interested cell types activation upon different stimulation. All above research were carried out in whole PBMCs. These findings were partially supported by different studies. Mian and colleague have shown that in PBMCs from healthy non-smokers, the expression of CD69, a key activation marker for NK cells, was significantly inhibited when PBMCs was treated by cigarette

smoke after poly I:C activation (Mian et al., 2009). Hogan *et al* demonstrated that CSE significantly reduced the cytokine production and the ability to kill target cells of $\alpha$-GalCel activated invariant NKT cells in PBMCs from healthy non-smoking participants. Meanwhile, the activation marker of CD107, but not CD69, was decreased (Hogan et al., 2011).

CSE suppression effects on isolated NKT-like cells ($CD3^+CD56^+$) and NK cells ($CD3^-CD56^+$) in response to IL-15 were also compared between groups. We found that, *in vitro*, CSE significantly suppresses the activation levels of IL-15 treated NKT-like cells and NK cells isolated from PBMCs in healthy non-smokers and smokes with or without COPD. However, this effect on NK cells was much more significant in cuurent smokers with COPD. Previous study has shown that cigarette smoke inhibited IL-12 and type-1 IFN production in activated PBMCs, which consequently decreased the IL-15 levels in healthy non-smokers (Mian et al., 2008, Mian et al., 2009). Also, both *in vitro* and *in vivo*, cigarette smoke significantly diminished IL-15 mRNA levels in poly I:C-activated PBMCs from healthy non-smoking participants. IL-15 production was markedly impaired in smoke-conditioned medium treated PBMCs upon poly I:C simulation.

Granzymes, perforin and Fas-Fas ligand are three major killing molecules that facilitate NKT-like cells and NK cells performing their cytotoxic functions. We found that expression of granzyme B on IL-15 treated NKT-like cells and NK cells were markedly reduced by CSE in PBMCs from healthy non-smokers. Also, CSE significantly suppressed granzyme B expression by NK cells in response to IL-15 in current smokers with COPD. Surprisingly, CSE did not induce any remarkable impacts on expression levels of perforin and Fas-ligand in all groups. Perforin was highly expressed in all stimulating conditions whereas Fas-L showed very low levels. These findings were partially supported by other research. Morissette and colleague illustrated that no statistical differences were observed on the expression of perforin and granzyme B in peripheral NK cells from either healthy volunteers or smokers with evidence of emphysema. Protein expression of Fas-ligand was not detected by flow cytometry (Morissette et al., 2007). The cytotoxicities of NK cells and NKT cells from PBMCs were both markedly reduced in COPD patients compared to healthy volunteers (Urbanowicz et al., 2009). *In vitro*, the percentage of perforin /granzyme B double positive NK cells was significantly lower in COPD patients compared to controls. Although there was a markedly increase in soluble Fas in plasma from severe COPD patients, there were no differences

on its expression in mild or moderate COPD and healthy controls (Yasuda et al., 1998). Hodge *et al* also demonstrated that there were no significant changes in the expression levels of granzyme A by NK or NKT-like cells in the blood of COPD patients compared to controls (Hodge et al., 2013). Taken together, our data suggest that granzyme B, but not perforin and Fas-ligand, was a functional molecule attributing to the significant impairment of NK cell killing ability in response to CSE in COPD patients. The action of granzyme B against target cells can be either perforin dependent (Bossi and Griffiths, 2005, Monks et al., 1998) or perforin-independent (Froelich et al., 1996, Metkar et al., 2002, Shi et al., 1997). No matter which action is undertaken, granzyme B mediated apoptosis is caspase-dependent (Talanian et al., 1997, Yang et al., 1998).

We also showed, *in vitro*, there were no significant differences on KIR (CD158e1) expression by $CD56^{+}$ with or without CSE treatment. KIR expression was at a very low level in each group. Few studies were carried out to detect the KIRs expression in either healthy volunteers or COPD patients. However, one study reported that NKG2D expression was not inhibited in cigarette smoke treated PBMCs after poly I:C induction (Mian et al., 2009). Therefore, KIR (CD158e1) was

not a functional inhibitory receptor for CSE induced suppression of $CD56^+$ cells activation in response to IL-15.

Although CSE significantly suppressed the isolated $CD56^+$ cell activation and their granzyme B expression in IL-15 conditioned medium in both healthy non-smokers and current smokers with COPD, there were no statistical differences on the absolute suppression levels between groups, which were different from our early findings that showed in un-isolated NK $CD56^+$ cells, absolute suppression levels induced by CSE on IL-15 mediated NK cell activation was significantly higher in current smokers with COPD compared to healthy non-smokers and current healthy smokers. Two reasons might be accounted for that. Firstly, small sample size was used in this study. In figure 3.3.7, the absolute suppreesion level induced by CSE on NK cell activation was higher than other two healthy groups, although no statistical differences were presented. Secondly, IL-15 is a potent cytokine with the ability of stimulating many kinds of immune cells, such as memory $CD4^+$ T cells, naive or memory $CD8^+$ T cells, DCs, NKT cells and NK cells, which can further secrete more cytokines and chemokines to induce adaptive immune responses (Anderson et al., 1995, Cosman et al., 1995). Therefore, in whole PBMCs

sample, IL-15 can further induce other immune response on un-isolated $CD56^+$ cells.

It is important to note that cigarette smoke comprises a complex mixture of more than 4000 chemicals, such as nicotine, carbon monoxide, and tar (Hoffmann and Wynder, 1986, Stedman, 1968). Their immune-modulation effects on immune cells are distinct and also dose-dependent (Holt et al., 1976). To determine the specific component of cigarette smoke that regulates killer cell activation upon different stimulation is beyond the scope of our studies.

Several limitations should be considered in the whole project. Firstly, in the *ex vivo* study, there are many kinds of inhibitory and activating receptors expressed on killer cells but due to limited cell numbers we analyse two representative receptors that give an indication of the killing function of these cells. Secondly, we could not obtain healthy samples in our BAL study. Thirdly, *in vitro*, only small numbers of subjects were recruited. Moreover, there are many cytotoxic granules such as granzyme A and granzyme K related to killing mechanisms of $CD56^+$ cells. However, proportions of NKT-like cells and NK cells are very low in PBMCs. We could just detect

representative killing molecules in our research due to cell numbers.

In conclusion, five important findings have been illustrated in this study. Firstly, we showed for the first time that, *ex vivo*, killer cells, including $CD8^+$ T cells, NKT-like cells and NK cells, were significantly activated in current smokers with or without COPD compared to healthy non-smokers. Secondly, KIR (CD158e1) expression by all killer cell types was dramatically lower in smokers with or without COPD in comparison with healthy non-smokers. Thirdly, the cytotoxicity of $CD8^+$ T cells from COPD patients were significantly less than that in healthy volunteers. Forthly, *in vitro,* CSE markedly decreased IL-15 treated NK cell activation in current smokers with COPD compared to other three groups. Finally, the expression of granzyme B, but not perforin and Fas-L, was also significantly inhibited on IL-15 stimulated NK cells when CSE was added.

Over activation of killer cells provide more understanding on the pathology of COPD development. Abnormal expression of KIR also suggests the potential use of KIRs therapy in COPD. Less cytotoxicity of $CD8^+$ T cells may explain why lung tissue is susceptible to infection in COPD patients. Suppression effects induced by CSE on NK cell activation and killing

molecule expression indicate that chronic exposure to cigarette smoke leads to not only cellular death and damage in the airways, but also failure to activate critical intracellular signalling mechanisms that initiate protective host defence. The consequence is dysfunction of various cell types with accompanying predisposition to respiratory infection.

# References

AARON, S. D., ANGEL, J. B., LUNAU, M., WRIGHT, K., FEX, C., LE SAUX, N. & DALES, R. E. (2001) Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 163**,** 349-55.

ABBOUD, R. T. & VIMALANATHAN, S. (2008) Pathogenesis of COPD. Part I. The role of protease-antiprotease imbalance in emphysema. *Int J Tuberc Lung Dis,* 12**,** 361-7.

ABES, R., DUTERTRE, C. A., AGNELLI, L. & TEILLAUD, J. L. (2009) Activating and inhibitory Fcgamma receptors in immunotherapy: being the actor or being the target. *Expert Rev Clin Immunol,* 5**,** 735-47.

AGGARWAL, B. B. (2003) Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol,* 3**,** 745-56.

AGUSTI, A. (2007) Systemic effects of chronic obstructive pulmonary disease: what we know and what we don't know (but should). *Proc Am Thorac Soc,* 4**,** 522-5.

AGUSTI, A. G., SAULEDA, J., MIRALLES, C., GOMEZ, C., TOGORES, B., SALA, E., BATLE, S. & BUSQUETS, X. (2002) Skeletal muscle apoptosis and weight loss in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 166**,** 485-9.

AICHER, A., HEESCHEN, C., MOHAUPT, M., COOKE, J. P., ZEIHER, A. M. & DIMMELER, S. (2003) Nicotine strongly activates dendritic cell-mediated adaptive immunity: potential role for progression of atherosclerotic lesions. *Circulation,* 107**,** 604-11.

ALAM, R. & GORSKA, M. (2003) 3. Lymphocytes. *J Allergy Clin Immunol,* 111**,** S476-85.

ALDONYTE, R., JANSSON, L., PIITULAINEN, E. & JANCIAUSKIENE, S. (2003) Circulating monocytes from healthy individuals and COPD patients. *Respir Res,* 4**,** 11.

ALMIRALL, J., GONZALEZ, C. A., BALANZO, X. & BOLIBAR, I. (1999) Proportion of community-acquired pneumonia cases attributable to tobacco smoking. *Chest,* 116**,** 375-9.

ALON, T., HEMO, I., ITIN, A., PE'ER, J., STONE, J. & KESHET, E. (1995) Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med,* 1**,** 1024-8.

AMMAR, M., MOKNI, M., BOUBAKER, S., EL GAIED, A., BEN OSMAN, A. & LOUZIR, H. (2008) Involvement of granzyme B and granulysin in the cytotoxic response in lichen planus. *J Cutan Pathol,* 35**,** 630-4.

ANDERSON, D. M., KUMAKI, S., AHDIEH, M., BERTLES, J., TOMETSKO, M., LOOMIS, A., GIRI, J., COPELAND, N. G., GILBERT, D. J., JENKINS, N. A. & ET AL. (1995) Functional characterization of the human interleukin-15 receptor alpha chain and close linkage of IL15RA and IL2RA genes. *J Biol Chem,* 270**,** 29862-9.

ANDERSON, D. M., LYMAN, S. D., BAIRD, A., WIGNALL, J. M., EISENMAN, J., RAUCH, C., MARCH, C. J., BOSWELL, H. S., GIMPEL, S. D., COSMAN, D. & ET AL. (1990) Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell,* 63**,** 235-43.

ARASE, H., ARASE, N., KOBAYASHI, Y., NISHIMURA, Y., YONEHARA, S. & ONOE, K. (1994) Cytotoxicity of fresh NK1.1+ T cell receptor

alpha/beta+ thymocytes against a CD4+8+ thymocyte population associated with intact Fas antigen expression on the target. *J Exp Med,* 180**,** 423-32.

ARCAVI, L. & BENOWITZ, N. L. (2004) Cigarette smoking and infection. *Arch Intern Med,* 164**,** 2206-16.

ARDAVIN, C. & SHORTMAN, K. (1992) Cell surface marker analysis of mouse thymic dendritic cells. *Eur J Immunol,* 22**,** 859-62.

ARGAST, G. M., CAMPBELL, J. S., BROOLING, J. T. & FAUSTO, N. (2004) Epidermal growth factor receptor transactivation mediates tumor necrosis factor-induced hepatocyte replication. *J Biol Chem,* 279**,** 34530-6.

ASAO, H., OKUYAMA, C., KUMAKI, S., ISHII, N., TSUCHIYA, S., FOSTER, D. & SUGAMURA, K. (2001) Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. *J Immunol,* 167**,** 1-5.

ATKINSON, J. J. & SENIOR, R. M. (2003) Matrix metalloproteinase-9 in lung remodeling. *Am J Respir Cell Mol Biol,* 28**,** 12-24.

AVISSAR, N., FINKELSTEIN, J. N., HOROWITZ, S., WILLEY, J. C., COY, E., FRAMPTON, M. W., WATKINS, R. H., KHULLAR, P., XU, Y. L. & COHEN, H. J. (1996) Extracellular glutathione peroxidase in human lung epithelial lining fluid and in lung cells. *Am J Physiol,* 270**,** L173-82.

BABUSYTE, A., STRAVINSKAITE, K., JEROCH, J., LOTVALL, J., SAKALAUSKAS, R. & SITKAUSKIENE, B. (2007) Patterns of airway inflammation and MMP-12 expression in smokers and ex-smokers with COPD. *Respir Res,* 8**,** 81.

BACAKOGLU, F., ATASEVER, A., OZHAN, M. H., GURGUN, C., OZKILIC, H. & GUZELANT, A. (2003) Plasma and bronchoalveolar lavage fluid levels of endothelin-1 in patients with chronic obstructive pulmonary disease and pulmonary hypertension. *Respiration,* 70**,** 594-9.

BAETU, T. M. & HISCOTT, J. (2002) On the TRAIL to apoptosis. *Cytokine Growth Factor Rev,* 13**,** 199-207.

BALBI, B., BASON, C., BALLEARI, E., FIASELLA, F., PESCI, A., GHIO, R. & FABIANO, F. (1997) Increased bronchoalveolar granulocytes and granulocyte/macrophage colony-stimulating factor during exacerbations of chronic bronchitis. *Eur Respir J,* 10**,** 846-50.

BALMES, J., BECKLAKE, M., BLANC, P., HENNEBERGER, P., KREISS, K., MAPP, C., MILTON, D., SCHWARTZ, D., TOREN, K. & VIEGI, G. (2003) American Thoracic Society Statement: Occupational contribution to the burden of airway disease. *Am J Respir Crit Care Med,* 167**,** 787-97.

BALZANO, G., STEFANELLI, F., IORIO, C., DE FELICE, A., MELILLO, E. M., MARTUCCI, M. & MELILLO, G. (1999) Eosinophilic inflammation in stable chronic obstructive pulmonary disease. Relationship with neutrophils and airway function. *Am J Respir Crit Care Med,* 160**,** 1486-92.

BAMFORD, R. N., BATTIATA, A. P. & WALDMANN, T. A. (1996) IL-15: the role of translational regulation in their expression. *J Leukoc Biol,* 59**,** 476-80.

BANCHEREAU, J., BRIERE, F., CAUX, C., DAVOUST, J., LEBECQUE, S., LIU, Y. J., PULENDRAN, B. & PALUCKA, K. (2000) Immunobiology of dendritic cells. *Annu Rev Immunol,* 18**,** 767-811.

BARALDO, S., TURATO, G., BADIN, C., BAZZAN, E., BEGHE, B., ZUIN, R., CALABRESE, F., CASONI, G., MAESTRELLI, P., PAPI, A., FABBRI, L. M. & SAETTA, M. (2004) Neutrophilic infiltration within the airway smooth muscle in patients with COPD. *Thorax,* 59**,** 308-12.

BARBERS, R. G., EVANS, M. J., GONG, H., JR. & TASHKIN, D. P. (1991) Enhanced alveolar monocytic phagocyte (macrophage) proliferation in tobacco and marijuana smokers. *Am Rev Respir Dis,* 143**,** 1092-5.

BARCELO, B., PONS, J., FERRER, J. M., SAULEDA, J., FUSTER, A. & AGUSTI, A. G. (2008) Phenotypic characterisation of T-lymphocytes in COPD: abnormal CD4+CD25+ regulatory T-lymphocyte response to tobacco smoking. *Eur Respir J,* 31**,** 555-62.

BARCZYK, A., PIERZCHALA, W., KON, O. M., COSIO, B., ADCOCK, I. M. & BARNES, P. J. (2006) Cytokine production by bronchoalveolar lavage T lymphocytes in chronic obstructive pulmonary disease. *J Allergy Clin Immunol,* 117**,** 1484-92.

BARKER, D. J., GODFREY, K. M., FALL, C., OSMOND, C., WINTER, P. D. & SHAHEEN, S. O. (1991) Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease. *Bmj,* 303**,** 671-5.

BARNES, P. J. (2000) Chronic obstructive pulmonary disease. *N Engl J Med,* 343**,** 269-80.

BARNES, P. J. (2001) IL-10: a key regulator of allergic disease. *Clin Exp Allergy,* 31**,** 667-9.

BARNES, P. J. (2002) New treatments for COPD. *Nat Rev Drug Discov,* 1**,** 437-46.

BARNES, P. J. (2004a) Alveolar macrophages as orchestrators of COPD. *Copd,* 1**,** 59-70.

BARNES, P. J. (2004b) Alveolar macrophages in chronic obstructive pulmonary disease (COPD). *Cell Mol Biol (Noisy-le-grand),* 50 Online Pub**,** OL627-37.

BARNES, P. J. (2004c) Mediators of chronic obstructive pulmonary disease. *Pharmacological reviews,* 56**,** 515-48.

BARNES, P. J. (2004d) Mediators of chronic obstructive pulmonary disease. *Pharmacol Rev,* 56**,** 515-48.

BARNES, P. J. (2007) New molecular targets for the treatment of neutrophilic diseases. *J Allergy Clin Immunol,* 119**,** 1055-62; quiz 1063-4.

BARNES, P. J. (2009) The cytokine network in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol,* 41**,** 631-8.

BARNES, P. J., CHOWDHURY, B., KHARITONOV, S. A., MAGNUSSEN, H., PAGE, C. P., POSTMA, D. & SAETTA, M. (2006) Pulmonary biomarkers in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 174**,** 6-14.

BARNES, T. C., ANDERSON, M. E. & MOOTS, R. J. (2011) The many faces of interleukin-6: the role of IL-6 in inflammation, vasculopathy, and fibrosis in systemic sclerosis. *Int J Rheumatol,* 2011**,** 721608.

BARRY, M. & BLEACKLEY, R. C. (2002) Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol,* 2**,** 401-9.

BATTAGLIA, S., MAUAD, T., VAN SCHADEWIJK, A. M., VIGNOLA, A. M., RABE, K. F., BELLIA, V., STERK, P. J. & HIEMSTRA, P. S. (2007) Differential distribution of inflammatory cells in large and small airways in smokers. *J Clin Pathol,* 60**,** 907-11.

BAUER, S., GROH, V., WU, J., STEINLE, A., PHILLIPS, J. H., LANIER, L. L. & SPIES, T. (1999) Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science,* 285**,** 727-9.

BAZZONI, F. & BEUTLER, B. (1996) The tumor necrosis factor ligand and receptor families. *N Engl J Med,* 334**,** 1717-25.

BAZZONI, F., CASSATELLA, M. A., ROSSI, F., CESKA, M., DEWALD, B. & BAGGIOLINI, M. (1991) Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. *J Exp Med,* 173**,** 771-4.

BECK, S. & BARRELL, B. G. (1988) Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens. *Nature,* 331**,** 269-72.

BEEH, K. M., KORNMANN, O., BUHL, R., CULPITT, S. V., GIEMBYCZ, M. A. & BARNES, P. J. (2003) Neutrophil chemotactic activity of sputum from patients with COPD: role of interleukin 8 and leukotriene B4. *Chest,* 123**,** 1240-7.

BENDELAC, A. (1995) Mouse NK1+ T cells. *Curr Opin Immunol,* 7**,** 367-74.

BENDELAC, A., RIVERA, M. N., PARK, S. H. & ROARK, J. H. (1997) Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu Rev Immunol,* 15**,** 535-62.

BENNOUNA, S., BLISS, S. K., CURIEL, T. J. & DENKERS, E. Y. (2003) Cross-talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection. *J Immunol,* 171**,** 6052-8.

BERENSON, C. S., GARLIPP, M. A., GROVE, L. J., MALONEY, J. & SETHI, S. (2006) Impaired phagocytosis of nontypeable Haemophilus influenzae by human alveolar macrophages in chronic obstructive pulmonary disease. *J Infect Dis,* 194**,** 1375-84.

BERENSON, C. S., KRUZEL, R. L., EBERHARDT, E. & SETHI, S. (2013) Phagocytic dysfunction of human alveolar macrophages and severity of chronic obstructive pulmonary disease. *J Infect Dis,* 208**,** 2036-45.

BERTONE, S., SCHIAVETTI, F., BELLOMO, R., VITALE, C., PONTE, M., MORETTA, L. & MINGARI, M. C. (1999) Transforming growth factor-beta-induced expression of CD94/NKG2A inhibitory receptors in human T lymphocytes. *Eur J Immunol,* 29**,** 23-9.

BERZINS, S. P., ULDRICH, A. P., PELLICCI, D. G., MCNAB, F., HAYAKAWA, Y., SMYTH, M. J. & GODFREY, D. I. (2004) Parallels and distinctions between T and NKT cell development in the thymus. *Immunol Cell Biol,* 82**,** 269-75.

BESSA, V., TSELIOU, E., BAKAKOS, P. & LOUKIDES, S. (2008) Noninvasive evaluation of airway inflammation in asthmatic patients who smoke: implications for application in clinical practice. *Ann Allergy Asthma Immunol,* 101**,** 226-32; quiz 232-4, 278.

BETSUYAKU, T., NISHIMURA, M., TAKEYABU, K., TANINO, M., MIYAMOTO, K. & KAWAKAMI, Y. (2000) Decline in FEV(1) in

community-based older volunteers with higher levels of neutrophil elastase in bronchoalveolar lavage fluid. *Respiration,* 67**,** 261-7.

BETSUYAKU, T., NISHIMURA, M., TAKEYABU, K., TANINO, M., VENGE, P., XU, S. & KAWAKAMI, Y. (1999) Neutrophil granule proteins in bronchoalveolar lavage fluid from subjects with subclinical emphysema. *Am J Respir Crit Care Med,* 159**,** 1985-91.

BIERNACKI, W. A., KHARITONOV, S. A. & BARNES, P. J. (2003) Increased leukotriene B4 and 8-isoprostane in exhaled breath condensate of patients with exacerbations of COPD. *Thorax,* 58**,** 294-8.

BITTERMAN, P. B., SALTZMAN, L. E., ADELBERG, S., FERRANS, V. J. & CRYSTAL, R. G. (1984) Alveolar macrophage replication. One mechanism for the expansion of the mononuclear phagocyte population in the chronically inflamed lung. *J Clin Invest,* 74**,** 460-9.

BLANCHARD, C. & ROTHENBERG, M. E. (2009) Biology of the eosinophil. *Adv Immunol,* 101**,** 81-121.

BLIDBERG, K., PALMBERG, L., DAHLEN, B., LANTZ, A. S. & LARSSON, K. (2012) Increased neutrophil migration in smokers with or without chronic obstructive pulmonary disease. *Respirology,* 17**,** 854-60.

BLUESTONE, J. A. & TANG, Q. (2005) How do CD4+CD25+ regulatory T cells control autoimmunity? *Curr Opin Immunol,* 17**,** 638-42.

BLUMENTHAL, R. L., CAMPBELL, D. E., HWANG, P., DEKRUYFF, R. H., FRANKEL, L. R. & UMETSU, D. T. (2001) Human alveolar macrophages induce functional inactivation in antigen-specific CD4 T cells. *J Allergy Clin Immunol,* 107**,** 258-64.

BORCHERS, M. T., CARTY, M. P. & LEIKAUF, G. D. (1999) Regulation of human airway mucins by acrolein and inflammatory mediators. *Am J Physiol,* 276**,** L549-55.

BORREGAARD, N., SORENSEN, O. E. & THEILGAARD-MONCH, K. (2007) Neutrophil granules: a library of innate immunity proteins. *Trends Immunol,* 28**,** 340-5.

BOSMANN, M. & WARD, P. A. (2013) Modulation of inflammation by interleukin-27. *J Leukoc Biol*.

BOSSI, G. & GRIFFITHS, G. M. (2005) CTL secretory lysosomes: biogenesis and secretion of a harmful organelle. *Semin Immunol,* 17**,** 87-94.

BOUALLEGUE, A., DAOU, G. B. & SRIVASTAVA, A. K. (2007) Endothelin-1-induced signaling pathways in vascular smooth muscle cells. *Curr Vasc Pharmacol,* 5**,** 45-52.

BOWMAN, E. P., CAMPBELL, J. J., DRUEY, K. M., SCHESCHONKA, A., KEHRL, J. H. & BUTCHER, E. C. (1998) Regulation of chemotactic and proadhesive responses to chemoattractant receptors by RGS (regulator of G-protein signaling) family members. *J Biol Chem,* 273**,** 28040-8.

BOYSON, J. E., ERSKINE, R., WHITMAN, M. C., CHIU, M., LAU, J. M., KOOPMAN, L. A., VALTER, M. M., ANGELISOVA, P., HOREJSI, V. & STROMINGER, J. L. (2002) Disulfide bond-mediated dimerization of HLA-G on the cell surface. *Proc Natl Acad Sci U S A,* 99**,** 16180-5.

BOZZA, P. T., YU, W., PENROSE, J. F., MORGAN, E. S., DVORAK, A. M. & WELLER, P. F. (1997) Eosinophil lipid bodies: specific, inducible

intracellular sites for enhanced eicosanoid formation. *J Exp Med,* 186**,** 909-20.

BRAMAN, S. S. (2006) The global burden of asthma. *Chest,* 130**,** 4S-12S.

BRATKE, K., KLUG, A., JULIUS, P., KUEPPER, M., LOMMATZSCH, M., SPARMANN, G., LUTTMANN, W. & VIRCHOW, J. C. (2008) Granzyme K: a novel mediator in acute airway inflammation. *Thorax,* 63**,** 1006-11.

BRAUD, V. M., ALLAN, D. S., O'CALLAGHAN, C. A., SODERSTROM, K., D'ANDREA, A., OGG, G. S., LAZETIC, S., YOUNG, N. T., BELL, J. I., PHILLIPS, J. H., LANIER, L. L. & MCMICHAEL, A. J. (1998) HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature,* 391**,** 795-9.

BRICKSON, S., JI, L. L., SCHELL, K., OLABISI, R., ST PIERRE SCHNEIDER, B. & BEST, T. M. (2003) M1/70 attenuates blood-borne neutrophil oxidants, activation, and myofiber damage following stretch injury. *J Appl Physiol (1985),* 95**,** 969-76.

BRIGHTLING, C. E., MCKENNA, S., HARGADON, B., BIRRING, S., GREEN, R., SIVA, R., BERRY, M., PARKER, D., MONTEIRO, W., PAVORD, I. D. & BRADDING, P. (2005) Sputum eosinophilia and the short term response to inhaled mometasone in chronic obstructive pulmonary disease. *Thorax,* 60**,** 193-8.

BRIGHTLING, C. E., WARD, R., WOLTMANN, G., BRADDING, P., SHELLER, J. R., DWORSKI, R. & PAVORD, I. D. (2000) Induced sputum inflammatory mediator concentrations in eosinophilic bronchitis and asthma. *Am J Respir Crit Care Med,* 162**,** 878-82.

BRINDICCI, C., ITO, K., RESTA, O., PRIDE, N. B., BARNES, P. J. & KHARITONOV, S. A. (2005) Exhaled nitric oxide from lung periphery is increased in COPD. *Eur Respir J,* 26**,** 52-9.

BROOKS, S. M., BAKER, D. B., GANN, P. H., JARABEK, A. M., HERTZBERG, V., GALLAGHER, J., BIAGINI, R. E. & BERNSTEIN, I. L. (1990) Cold air challenge and platinum skin reactivity in platinum refinery workers. Bronchial reactivity precedes skin prick response. *Chest,* 97**,** 1401-7.

BROWN, C. A., CROMBIE, I. K. & TUNSTALL-PEDOE, H. (1994) Failure of cigarette smoking to explain international differences in mortality from chronic obstructive pulmonary disease. *J Epidemiol Community Health,* 48**,** 134-9.

BROWN, J. M., WILSON, T. M. & METCALFE, D. D. (2008) The mast cell and allergic diseases: role in pathogenesis and implications for therapy. *Clin Exp Allergy,* 38**,** 4-18.

BRYCESON, Y. T., MARCH, M. E., BARBER, D. F., LJUNGGREN, H. G. & LONG, E. O. (2005) Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J Exp Med,* 202**,** 1001-12.

BUCCHIONI, E., KHARITONOV, S. A., ALLEGRA, L. & BARNES, P. J. (2003) High levels of interleukin-6 in the exhaled breath condensate of patients with COPD. *Respir Med,* 97**,** 1299-302.

BUIST, A. S., MCBURNIE, M. A., VOLLMER, W. M., GILLESPIE, S., BURNEY, P., MANNINO, D. M., MENEZES, A. M., SULLIVAN, S. D., LEE, T. A., WEISS, K. B., JENSEN, R. L., MARKS, G. B.,

GULSVIK, A. & NIZANKOWSKA-MOGILNICKA, E. (2007) International variation in the prevalence of COPD (the BOLD Study): a population-based prevalence study. *Lancet,* 370**,** 741-50.

BULFONE-PAUS, S., BULANOVA, E., POHL, T., BUDAGIAN, V., DURKOP, H., RUCKERT, R., KUNZENDORF, U., PAUS, R. & KRAUSE, H. (1999) Death deflected: IL-15 inhibits TNF-alpha-mediated apoptosis in fibroblasts by TRAF2 recruitment to the IL-15Ralpha chain. *Faseb J,* 13**,** 1575-85.

BURDIN, N. & KRONENBERG, M. (1999) CD1-mediated immune responses to glycolipids. *Curr Opin Immunol,* 11**,** 326-31.

BURGEL, P. R. & NADEL, J. A. (2004) Roles of epidermal growth factor receptor activation in epithelial cell repair and mucin production in airway epithelium. *Thorax,* 59**,** 992-6.

BURROWS, B., KNUDSON, R. J., CLINE, M. G. & LEBOWITZ, M. D. (1977) Quantitative relationships between cigarette smoking and ventilatory function. *Am Rev Respir Dis,* 115**,** 195-205.

CALABRESE, F., BARALDO, S., BAZZAN, E., LUNARDI, F., REA, F., MAESTRELLI, P., TURATO, G., LOKAR-OLIANI, K., PAPI, A., ZUIN, R., SFRISO, P., BALESTRO, E., DINARELLO, C. A. & SAETTA, M. (2008) IL-32, a novel proinflammatory cytokine in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 178**,** 894-901.

CALIGIURI, M. A., MURRAY, C., ROBERTSON, M. J., WANG, E., COCHRAN, K., CAMERON, C., SCHOW, P., ROSS, M. E., KLUMPP, T. R., SOIFFER, R. J. & ET AL. (1993) Selective modulation of human natural killer cells in vivo after prolonged infusion of low dose recombinant interleukin 2. *J Clin Invest,* 91**,** 123-32.

CALIKOGLU, M., SAHIN, G., UNLU, A., OZTURK, C., TAMER, L., ERCAN, B., KANIK, A. & ATIK, U. (2004) Leptin and TNF-alpha levels in patients with chronic obstructive pulmonary disease and their relationship to nutritional parameters. *Respiration,* 71**,** 45-50.

CALL, M. E. & WUCHERPFENNIG, K. W. (2004) Molecular mechanisms for the assembly of the T cell receptor-CD3 complex. *Mol Immunol,* 40**,** 1295-305.

CANTIN, A. M., FELLS, G. A., HUBBARD, R. C. & CRYSTAL, R. G. (1990) Antioxidant macromolecules in the epithelial lining fluid of the normal human lower respiratory tract. *J Clin Invest,* 86**,** 962-71.

CANTIN, A. M., HANRAHAN, J. W., BILODEAU, G., ELLIS, L., DUPUIS, A., LIAO, J., ZIELENSKI, J. & DURIE, P. (2006) Cystic fibrosis transmembrane conductance regulator function is suppressed in cigarette smokers. *Am J Respir Crit Care Med,* 173**,** 1139-44.

CANTONI, C., VERDIANI, S., FALCO, M., PESSINO, A., CILLI, M., CONTE, R., PENDE, D., PONTE, M., MIKAELSSON, M. S., MORETTA, L. & BIASSONI, R. (1998) p49, a putative HLA class I-specific inhibitory NK receptor belonging to the immunoglobulin superfamily. *Eur J Immunol,* 28**,** 1980-90.

CAPELLI, A., DI STEFANO, A., GNEMMI, I., BALBO, P., CERUTTI, C. G., BALBI, B., LUSUARDI, M. & DONNER, C. F. (1999) Increased

MCP-1 and MIP-1beta in bronchoalveolar lavage fluid of chronic bronchitics. *Eur Respir J,* 14**,** 160-5.

CARSON, W. E., ROSS, M. E., BAIOCCHI, R. A., MARIEN, M. J., BOIANI, N., GRABSTEIN, K. & CALIGIURI, M. A. (1995) Endogenous production of interleukin 15 by activated human monocytes is critical for optimal production of interferon-gamma by natural killer cells in vitro. *J Clin Invest,* 96**,** 2578-82.

CARTER, A. B., MONICK, M. M. & HUNNINGHAKE, G. W. (1998) Lipopolysaccharide-induced NF-kappaB activation and cytokine release in human alveolar macrophages is PKC-independent and TK- and PC-PLC-dependent. *Am J Respir Cell Mol Biol,* 18**,** 384-91.

CARTER, R. I., UNGURS, M. J., MUMFORD, R. A. & STOCKLEY, R. A. (2012) Aalpha-Val360: a marker of neutrophil elastase and COPD disease activity. *Eur Respir J,* 41**,** 31-8.

CARUSO, A., LICENZIATI, S., CORULLI, M., CANARIS, A. D., DE FRANCESCO, M. A., FIORENTINI, S., PERONI, L., FALLACARA, F., DIMA, F., BALSARI, A. & TURANO, A. (1997) Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. *Cytometry,* 27**,** 71-6.

CASOLARO, M. A., BERNAUDIN, J. F., SALTINI, C., FERRANS, V. J. & CRYSTAL, R. G. (1988) Accumulation of Langerhans' cells on the epithelial surface of the lower respiratory tract in normal subjects in association with cigarette smoking. *Am Rev Respir Dis,* 137**,** 406-11.

CASTRICONI, R., CANTONI, C., DELLA CHIESA, M., VITALE, M., MARCENARO, E., CONTE, R., BIASSONI, R., BOTTINO, C., MORETTA, L. & MORETTA, A. (2003) Transforming growth factor beta 1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells. *Proc Natl Acad Sci U S A,* 100**,** 4120-5.

CASTRICONI, R., DONDERO, A., CORRIAS, M. V., LANINO, E., PENDE, D., MORETTA, L., BOTTINO, C. & MORETTA, A. (2004) Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: critical role of DNAX accessory molecule-1-poliovirus receptor interaction. *Cancer Res,* 64**,** 9180-4.

CATALDO, D., MUNAUT, C., NOEL, A., FRANKENNE, F., BARTSCH, P., FOIDART, J. M. & LOUIS, R. (2000) MMP-2- and MMP-9-linked gelatinolytic activity in the sputum from patients with asthma and chronic obstructive pulmonary disease. *Int Arch Allergy Immunol,* 123**,** 259-67.

CEDERBOM, L., HALL, H. & IVARS, F. (2000) CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur J Immunol,* 30**,** 1538-43.

CELLI, B. R., COTE, C. G., MARIN, J. M., CASANOVA, C., MONTES DE OCA, M., MENDEZ, R. A., PINTO PLATA, V. & CABRAL, H. J. (2004) The body-mass index, airflow obstruction, dyspnea, and exercise capacity index in chronic obstructive pulmonary disease. *N Engl J Med,* 350**,** 1005-12.

CELLI, B. R. & MACNEE, W. (2004) Standards for the diagnosis and treatment of patients with COPD: a summary of the ATS/ERS position paper. *Eur Respir J,* 23**,** 932-46.

CERWENKA, A., BARON, J. L. & LANIER, L. L. (2001) Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc Natl Acad Sci U S A,* 98**,** 11521-6.

CHAKRABARTI, B., CALVERLEY, P. M. & DAVIES, P. D. (2007) Tuberculosis and its incidence, special nature, and relationship with chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis,* 2**,** 263-72.

CHALMERS, G. W., MACLEOD, K. J., SRIRAM, S., THOMSON, L. J., MCSHARRY, C., STACK, B. H. & THOMSON, N. C. (1999) Sputum endothelin-1 is increased in cystic fibrosis and chronic obstructive pulmonary disease. *Eur Respir J,* 13**,** 1288-92.

CHAPMAN, K. R., MANNINO, D. M., SORIANO, J. B., VERMEIRE, P. A., BUIST, A. S., THUN, M. J., CONNELL, C., JEMAL, A., LEE, T. A., MIRAVITLLES, M., ALDINGTON, S. & BEASLEY, R. (2006) Epidemiology and costs of chronic obstructive pulmonary disease. *Eur Respir J,* 27**,** 188-207.

CHAPMAN, R. W., PHILLIPS, J. E., HIPKIN, R. W., CURRAN, A. K., LUNDELL, D. & FINE, J. S. (2009) CXCR2 antagonists for the treatment of pulmonary disease. *Pharmacol Ther,* 121**,** 55-68.

CHAPMAN, T. L., HEIKEMAN, A. P. & BJORKMAN, P. J. (1999) The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. *Immunity,* 11**,** 603-13.

CHEN, G., SHANKAR, P., LANGE, C., VALDEZ, H., SKOLNIK, P. R., WU, L., MANJUNATH, N. & LIEBERMAN, J. (2001) CD8 T cells specific for human immunodeficiency virus, Epstein-Barr virus, and cytomegalovirus lack molecules for homing to lymphoid sites of infection. *Blood,* 98**,** 156-64.

CHEN, Z. & O'SHEA, J. J. (2008) Th17 cells: a new fate for differentiating helper T cells. *Immunol Res,* 41**,** 87-102.

CHERTOV, O., UEDA, H., XU, L. L., TANI, K., MURPHY, W. J., WANG, J. M., HOWARD, O. M., SAYERS, T. J. & OPPENHEIM, J. J. (1997) Identification of human neutrophil-derived cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils. *J Exp Med,* 186**,** 739-47.

CHI, S. Y., BAN, H. J., KWON, Y. S., OH, I. J., KIM, K. S., KIM, Y. I., KIM, Y. C. & LIM, S. C. (2011) Invariant natural killer T cells in chronic obstructive pulmonary disease. *Respirology,* 17**,** 486-92.

CHODNIEWICZ, D. & ZHELEV, D. V. (2003) Novel pathways of F-actin polymerization in the human neutrophil. *Blood,* 102**,** 2251-8.

CHOWDHURY, D. & LIEBERMAN, J. (2008) Death by a thousand cuts: granzyme pathways of programmed cell death. *Annu Rev Immunol,* 26**,** 389-420.

CHRYSOFAKIS, G., TZANAKIS, N., KYRIAKOY, D., TSOUMAKIDOU, M., TSILIGIANNI, I., KLIMATHIANAKI, M. & SIAFAKAS, N. M. (2004) Perforin expression and cytotoxic activity of sputum CD8+ lymphocytes in patients with COPD. *Chest,* 125**,** 71-6.

CHUNG, K. F. (2005) The role of airway smooth muscle in the pathogenesis of airway wall remodeling in chronic obstructive pulmonary disease. *Proc Am Thorac Soc,* 2**,** 347-54; discussion 371-2.

CHUNG, K. F. & ADCOCK, I. M. (2008) Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. *Eur Respir J,* 31**,** 1334-56.

CHURG, A., WANG, R. D., TAI, H., WANG, X., XIE, C., DAI, J., SHAPIRO, S. D. & WRIGHT, J. L. (2003) Macrophage metalloelastase mediates acute cigarette smoke-induced inflammation via tumor necrosis factor-alpha release. *Am J Respir Crit Care Med,* 167**,** 1083-9.

CHURG, A., WANG, R. D., TAI, H., WANG, X., XIE, C. & WRIGHT, J. L. (2004) Tumor necrosis factor-alpha drives 70% of cigarette smoke-induced emphysema in the mouse. *Am J Respir Crit Care Med,* 170**,** 492-8.

CLARK-LEWIS, I., MATTIOLI, I., GONG, J. H. & LOETSCHER, P. (2003) Structure-function relationship between the human chemokine receptor CXCR3 and its ligands. *J Biol Chem,* 278**,** 289-95.

COLONNA, M., NAVARRO, F., BELLON, T., LLANO, M., GARCIA, P., SAMARIDIS, J., ANGMAN, L., CELLA, M. & LOPEZ-BOTET, M. (1997) A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J Exp Med,* 186**,** 1809-18.

COLONNA, M. & SAMARIDIS, J. (1995) Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. *Science,* 268**,** 405-8.

COLONNA, M., SPIES, T., STROMINGER, J. L., CICCONE, E., MORETTA, A., MORETTA, L., PENDE, D. & VIALE, O. (1992) Alloantigen recognition by two human natural killer cell clones is associated with HLA-C or a closely linked gene. *Proc Natl Acad Sci U S A,* 89**,** 7983-5.

COOPER, M. A., FEHNIGER, T. A. & CALIGIURI, M. A. (2001a) The biology of human natural killer-cell subsets. *Trends Immunol,* 22**,** 633-40.

COOPER, M. A., FEHNIGER, T. A., TURNER, S. C., CHEN, K. S., GHAHERI, B. A., GHAYUR, T., CARSON, W. E. & CALIGIURI, M. A. (2001b) Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood,* 97**,** 3146-51.

CORINTI, S., ALBANESI, C., LA SALA, A., PASTORE, S. & GIROLOMONI, G. (2001) Regulatory activity of autocrine IL-10 on dendritic cell functions. *J Immunol,* 166**,** 4312-8.

CORRADI, M., MONTUSCHI, P., DONNELLY, L. E., PESCI, A., KHARITONOV, S. A. & BARNES, P. J. (2001) Increased nitrosothiols in exhaled breath condensate in inflammatory airway diseases. *Am J Respir Crit Care Med,* 163**,** 854-8.

COSIO, M., GHEZZO, H., HOGG, J. C., CORBIN, R., LOVELAND, M., DOSMAN, J. & MACKLEM, P. T. (1978) The relations between structural changes in small airways and pulmonary-function tests. *N Engl J Med,* 298**,** 1277-81.

COSMAN, D., FANGER, N., BORGES, L., KUBIN, M., CHIN, W., PETERSON, L. & HSU, M. L. (1997) A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules. *Immunity,* 7**,** 273-82.

COSMAN, D., KUMAKI, S., AHDIEH, M., EISENMAN, J., GRABSTEIN, K. H., PAXTON, R., DUBOSE, R., FRIEND, D., PARK, L. S., ANDERSON, D. & ET AL. (1995) Interleukin 15 and its receptor. *Ciba Found Symp,* 195**,** 221-9; discussion 229-33.

COSMAN, D., MULLBERG, J., SUTHERLAND, C. L., CHIN, W., ARMITAGE, R., FANSLOW, W., KUBIN, M. & CHALUPNY, N. J. (2001) ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity,* 14**,** 123-33.

COSTA, C., RUFINO, R., TRAVES, S. L., LAPA, E. S. J. R., BARNES, P. J. & DONNELLY, L. E. (2008) CXCR3 and CCR5 chemokines in induced sputum from patients with COPD. *Chest,* 133**,** 26-33.

CROOKS, S. W., BAYLEY, D. L., HILL, S. L. & STOCKLEY, R. A. (2000) Bronchial inflammation in acute bacterial exacerbations of chronic bronchitis: the role of leukotriene B4. *Eur Respir J,* 15**,** 274-80.

CROWE, N. Y., ULDRICH, A. P., KYPARISSOUDIS, K., HAMMOND, K. J., HAYAKAWA, Y., SIDOBRE, S., KEATING, R., KRONENBERG, M., SMYTH, M. J. & GODFREY, D. I. (2003) Glycolipid antigen drives rapid expansion and sustained cytokine production by NK T cells. *J Immunol,* 171**,** 4020-7.

CULPITT, S. V., ROGERS, D. F., SHAH, P., DE MATOS, C., RUSSELL, R. E., DONNELLY, L. E. & BARNES, P. J. (2003) Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 167**,** 24-31.

CULPITT, S. V., ROGERS, D. F., TRAVES, S. L., BARNES, P. J. & DONNELLY, L. E. (2005) Sputum matrix metalloproteases: comparison between chronic obstructive pulmonary disease and asthma. *Respir Med,* 99**,** 703-10.

D'ARMIENTO, J. M., SCHARF, S. M., ROTH, M. D., CONNETT, J. E., GHIO, A., STERNBERG, D., GOLDIN, J. G., LOUIS, T. A., MAO, J. T., O'CONNOR, G. T., RAMSDELL, J. W., RIES, A. L., SCHLUGER, N. W., SCIURBA, F. C., SKEANS, M. A., VOELKER, H., WALTER, R. E., WENDT, C. H., WEINMANN, G. G., WISE, R. A. & FORONJY, R. F. (2009) Eosinophil and T cell markers predict functional decline in COPD patients. *Respir Res,* 10**,** 113.

D'CRUZ, L. M. & KLEIN, L. (2005) Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol,* 6**,** 1152-9.

DAHLEN, I. & JANSON, C. (2002) Anxiety and depression are related to the outcome of emergency treatment in patients with obstructive pulmonary disease. *Chest,* 122**,** 1633-7.

DALL'OZZO, S., TARTAS, S., PAINTAUD, G., CARTRON, G., COLOMBAT, P., BARDOS, P., WATIER, H. & THIBAULT, G. (2004) Rituximab-dependent cytotoxicity by natural killer cells:

influence of FCGR3A polymorphism on the concentration-effect relationship. *Cancer Res,* 64**,** 4664-9.

DAMIANO, V. V., TSANG, A., KUCICH, U., ABRAMS, W. R., ROSENBLOOM, J., KIMBEL, P., FALLAHNEJAD, M. & WEINBAUM, G. (1986) Immunolocalization of elastase in human emphysematous lungs. *J Clin Invest,* 78**,** 482-93.

DANAHAY, H. & JACKSON, A. D. (2005) Epithelial mucus-hypersecretion and respiratory disease. *Curr Drug Targets Inflamm Allergy,* 4**,** 651-64.

DE BOER, W. I. (2005) Perspectives for cytokine antagonist therapy in COPD. *Drug Discov Today,* 10**,** 93-106.

DE BOER, W. I., SONT, J. K., VAN SCHADEWIJK, A., STOLK, J., VAN KRIEKEN, J. H. & HIEMSTRA, P. S. (2000) Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD. *J Pathol,* 190**,** 619-26.

DE GODOY, I., DONAHOE, M., CALHOUN, W. J., MANCINO, J. & ROGERS, R. M. (1996) Elevated TNF-alpha production by peripheral blood monocytes of weight-losing COPD patients. *Am J Respir Crit Care Med,* 153**,** 633-7.

DE SMEDT, T., VAN MECHELEN, M., DE BECKER, G., URBAIN, J., LEO, O. & MOSER, M. (1997) Effect of interleukin-10 on dendritic cell maturation and function. *Eur J Immunol,* 27**,** 1229-35.

DEFORGE, L. E., PRESTON, A. M., TAKEUCHI, E., KENNEY, J., BOXER, L. A. & REMICK, D. G. (1993) Regulation of interleukin 8 gene expression by oxidant stress. *J Biol Chem,* 268**,** 25568-76.

DEJACO, C., DUFTNER, C., GRUBECK-LOEBENSTEIN, B. & SCHIRMER, M. (2006) Imbalance of regulatory T cells in human autoimmune diseases. *Immunology,* 117**,** 289-300.

DEMEDTS, I. K., BRACKE, K. R., VAN POTTELBERGE, G., TESTELMANS, D., VERLEDEN, G. M., VERMASSEN, F. E., JOOS, G. F. & BRUSSELLE, G. G. (2007) Accumulation of dendritic cells and increased CCL20 levels in the airways of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 175**,** 998-1005.

DEMEDTS, I. K., BRUSSELLE, G. G., VERMAELEN, K. Y. & PAUWELS, R. A. (2005) Identification and characterization of human pulmonary dendritic cells. *Am J Respir Cell Mol Biol,* 32**,** 177-84.

DEMEDTS, I. K., MOREL-MONTERO, A., LEBECQUE, S., PACHECO, Y., CATALDO, D., JOOS, G. F., PAUWELS, R. A. & BRUSSELLE, G. G. (2006) Elevated MMP-12 protein levels in induced sputum from patients with COPD. *Thorax,* 61**,** 196-201.

DENBURG, J. A. (1999) Bone marrow in atopy and asthma: hematopoietic mechanisms in allergic inflammation. *Immunol Today,* 20**,** 111-3.

DENG, A., CHEN, S., LI, Q., LYU, S. C., CLAYBERGER, C. & KRENSKY, A. M. (2005) Granulysin, a cytolytic molecule, is also a chemoattractant and proinflammatory activator. *J Immunol,* 174**,** 5243-8.

DERRE, L., CORVAISIER, M., PANDOLFINO, M. C., DIEZ, E., JOTEREAU, F. & GERVOIS, N. (2002) Expression of CD94/NKG2-A on human T lymphocytes is induced by IL-12: implications for adoptive immunotherapy. *J Immunol,* 168**,** 4864-70.

DEVORA, R. A., ZLOTNIK. (2000) The biology of chemokines and their receptors. *Annu Rev Immunol,* 18**,** 217-242.

DI FRANCIA, M., BARBIER, D., MEGE, J. L. & OREHEK, J. (1994) Tumor necrosis factor-alpha levels and weight loss in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 150**,** 1453-5.

DI STEFANO, A., CAPELLI, A., LUSUARDI, M., BALBO, P., VECCHIO, C., MAESTRELLI, P., MAPP, C. E., FABBRI, L. M., DONNER, C. F. & SAETTA, M. (1998) Severity of airflow limitation is associated with severity of airway inflammation in smokers. *Am J Respir Crit Care Med,* 158**,** 1277-85.

DI STEFANO, A., CAPELLI, A., LUSUARDI, M., CARAMORI, G., BALBO, P., IOLI, F., SACCO, S., GNEMMI, I., BRUN, P., ADCOCK, I. M., BALBI, B., BARNES, P. J., CHUNG, K. F. & DONNER, C. F. (2001) Decreased T lymphocyte infiltration in bronchial biopsies of subjects with severe chronic obstructive pulmonary disease. *Clin Exp Allergy,* 31**,** 893-902.

DI STEFANO, A., CARAMORI, G., GNEMMI, I., CONTOLI, M., BRISTOT, L., CAPELLI, A., RICCIARDOLO, F. L., MAGNO, F., D'ANNA, S. E., ZANINI, A., CARBONE, M., SABATINI, F., USAI, C., BRUN, P., CHUNG, K. F., BARNES, P. J., PAPI, A., ADCOCK, I. M. & BALBI, B. (2009a) Association of increased CCL5 and CXCL7 chemokine expression with neutrophil activation in severe stable COPD. *Thorax,* 64**,** 968-75.

DI STEFANO, A., CARAMORI, G., GNEMMI, I., CONTOLI, M., VICARI, C., CAPELLI, A., MAGNO, F., D'ANNA, S. E., ZANINI, A., BRUN, P., CASOLARI, P., CHUNG, K. F., BARNES, P. J., PAPI, A., ADCOCK, I. & BALBI, B. (2009b) T helper type 17-related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients. *Clin Exp Immunol,* 157**,** 316-24.

DI STEFANO, A., CARAMORI, G., OATES, T., CAPELLI, A., LUSUARDI, M., GNEMMI, I., IOLI, F., CHUNG, K. F., DONNER, C. F., BARNES, P. J. & ADCOCK, I. M. (2002) Increased expression of nuclear factor-kappaB in bronchial biopsies from smokers and patients with COPD. *Eur Respir J,* 20**,** 556-63.

DI STEFANO, A., CARAMORI, G., RICCIARDOLO, F. L., CAPELLI, A., ADCOCK, I. M. & DONNER, C. F. (2004) Cellular and molecular mechanisms in chronic obstructive pulmonary disease: an overview. *Clin Exp Allergy,* 34**,** 1156-67.

DI STEFANO, A., MAESTRELLI, P., ROGGERI, A., TURATO, G., CALABRO, S., POTENA, A., MAPP, C. E., CIACCIA, A., COVACEV, L., FABBRI, L. M. & SAETTA, M. (1994) Upregulation of adhesion molecules in the bronchial mucosa of subjects with chronic obstructive bronchitis. *Am J Respir Crit Care Med,* 149**,** 803-10.

DIAMOND, M. S., STAUNTON, D. E., DE FOUGEROLLES, A. R., STACKER, S. A., GARCIA-AGUILAR, J., HIBBS, M. L. & SPRINGER, T. A. (1990) ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). *J Cell Biol,* 111**,** 3129-39.

DIEFENBACH, A., JENSEN, E. R., JAMIESON, A. M. & RAULET, D. H. (2001) Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature,* 413**,** 165-71.

DIEFENBACH, A., TOMASELLO, E., LUCAS, M., JAMIESON, A. M., HSIA, J. K., VIVIER, E. & RAULET, D. H. (2002) Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nat Immunol,* 3**,** 1142-9.

DOHERTY, D. G., NORRIS, S., MADRIGAL-ESTEBAS, L., MCENTEE, G., TRAYNOR, O., HEGARTY, J. E. & O'FARRELLY, C. (1999) The human liver contains multiple populations of NK cells, T cells, and CD3+CD56+ natural T cells with distinct cytotoxic activities and Th1, Th2, and Th0 cytokine secretion patterns. *J Immunol,* 163**,** 2314-21.

DOMAGALA-KULAWIK, J., HOSER, G., DABROWSKA, M. & CHAZAN, R. (2007) Increased proportion of Fas positive CD8+ cells in peripheral blood of patients with COPD. *Respir Med,* 101**,** 1338-43.

DONALDSON, G. C., SEEMUNGAL, T. A., PATEL, I. S., BHOWMIK, A., WILKINSON, T. M., HURST, J. R., MACCALLUM, P. K. & WEDZICHA, J. A. (2009) Airway and systemic inflammation and decline in lung function in patients with COPD. 2005. *Chest,* 136**,** e30.

DRAGONIERI, S., TONGOUSSOUVA, O., ZANINI, A., IMPERATORI, A. & SPANEVELLO, A. (2009) Markers of airway inflammation in pulmonary diseases assessed by induced sputum. *Monaldi Arch Chest Dis,* 71**,** 119-26.

DUARTE, N., STENSTROM, M., CAMPINO, S., BERGMAN, M. L., LUNDHOLM, M., HOLMBERG, D. & CARDELL, S. L. (2004) Prevention of diabetes in nonobese diabetic mice mediated by CD1d-restricted nonclassical NKT cells. *J Immunol,* 173**,** 3112-8.

DUBOIS, S., MARINER, J., WALDMANN, T. A. & TAGAYA, Y. (2002) IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells. *Immunity,* 17**,** 537-47.

EBERL, G. & MACDONALD, H. R. (1998) Rapid death and regeneration of NKT cells in anti-CD3epsilon- or IL-12-treated mice: a major role for bone marrow in NKT cell homeostasis. *Immunity,* 9**,** 345-53.

EISERICH, J. P., HRISTOVA, M., CROSS, C. E., JONES, A. D., FREEMAN, B. A., HALLIWELL, B. & VAN DER VLIET, A. (1998) Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature,* 391**,** 393-7.

EKBERG-JANSSON, A., ANDERSSON, B., AVRA, E., NILSSON, O. & LOFDAHL, C. G. (2000) The expression of lymphocyte surface antigens in bronchial biopsies, bronchoalveolar lavage cells and blood cells in healthy smoking and never-smoking men, 60 years old. *Respir Med,* 94**,** 264-72.

EKEOWA, U. I., GOOPTU, B., BELORGEY, D., HAGGLOF, P., KARLSSON-LI, S., MIRANDA, E., PEREZ, J., MACLEOD, I., KROGER, H., MARCINIAK, S. J., CROWTHER, D. C. & LOMAS, D. A. (2009) alpha1-Antitrypsin deficiency, chronic obstructive pulmonary disease and the serpinopathies. *Clin Sci (Lond),* 116**,** 837-50.

EMOTO, M. & KAUFMANN, S. H. (2003) Liver NKT cells: an account of heterogeneity. *Trends Immunol,* 24**,** 364-9.

ERIKSSON, S. (1965) Studies in alpha 1-antitrypsin deficiency. *Acta Med Scand Suppl,* 432**,** 1-85.

ERWIG, L. P. & HENSON, P. M. (2008) Clearance of apoptotic cells by phagocytes. *Cell Death Differ,* 15**,** 243-50.

ETHUIN, F., GERARD, B., BENNA, J. E., BOUTTEN, A., GOUGEREOT-POCIDALO, M. A., JACOB, L. & CHOLLET-MARTIN, S. (2004) Human neutrophils produce interferon gamma upon stimulation by interleukin-12. *Lab Invest,* 84**,** 1363-71.

EZZATI, M. (2005) Indoor air pollution and health in developing countries. *Lancet,* 366**,** 104-6.

FAIRCLOUGH, L., URBANOWICZ, R. A., CORNE, J. & LAMB, J. R. (2008) Killer cells in chronic obstructive pulmonary disease. *Clin Sci (Lond),* 114**,** 533-41.

FEHNIGER, T. A. & CALIGIURI, M. A. (2001) Ontogeny and expansion of human natural killer cells: clinical implications. *Int Rev Immunol,* 20**,** 503-34.

FELDMAN, C. & ANDERSON, R. (2013) Cigarette smoking and mechanisms of susceptibility to infections of the respiratory tract and other organ systems. *J Infect,* 67**,** 169-84.

FENG, J., GARRITY, D., CALL, M. E., MOFFETT, H. & WUCHERPFENNIG, K. W. (2005) Convergence on a distinctive assembly mechanism by unrelated families of activating immune receptors. *Immunity,* 22**,** 427-38.

FERLAZZO, G., THOMAS, D., LIN, S. L., GOODMAN, K., MORANDI, B., MULLER, W. A., MORETTA, A. & MUNZ, C. (2004) The abundant NK cells in human secondary lymphoid tissues require activation to express killer cell Ig-like receptors and become cytolytic. *J Immunol,* 172**,** 1455-62.

FERRINI, S., CAMBIAGGI, A., MEAZZA, R., SFORZINI, S., MARCIANO, S., MINGARI, M. C. & MORETTA, L. (1994) T cell clones expressing the natural killer cell-related p58 receptor molecule display heterogeneity in phenotypic properties and p58 function. *Eur J Immunol,* 24**,** 2294-8.

FERSON, M., EDWARDS, A., LIND, A., MILTON, G. W. & HERSEY, P. (1979) Low natural killer-cell activity and immunoglobulin levels associated with smoking in human subjects. *Int J Cancer,* 23**,** 603-9.

FINI, M. E., COOK, J. R., MOHAN, R. & BRINKERHOFF, C. E. (1998) regulation of matrix metalloproteinase gene expression in matrix metalloproteinases. *Academic Press, San Diego***,** 300-339.

FINKEL, T. (2003) Oxidant signals and oxidative stress. *Curr Opin Cell Biol,* 15**,** 247-54.

FINKELSTEIN, R., FRASER, R. S., GHEZZO, H. & COSIO, M. G. (1995) Alveolar inflammation and its relation to emphysema in smokers. *Am J Respir Crit Care Med,* 152**,** 1666-72.

FINLAY, G. A., O'DRISCOLL, L. R., RUSSELL, K. J., D'ARCY, E. M., MASTERSON, J. B., FITZGERALD, M. X. & O'CONNOR, C. M. (1997a) Matrix metalloproteinase expression and production by alveolar macrophages in emphysema. *Am J Respir Crit Care Med,* 156**,** 240-7.

FINLAY, G. A., RUSSELL, K. J., MCMAHON, K. J., D'ARCY E, M., MASTERSON, J. B., FITZGERALD, M. X. & O'CONNOR, C. M. (1997b) Elevated levels of matrix metalloproteinases in bronchoalveolar lavage fluid of emphysematous patients. *Thorax,* 52**,** 502-6.

FISCHER, B. M. & VOYNOW, J. A. (2002) Neutrophil elastase induces MUC5AC gene expression in airway epithelium via a pathway involving reactive oxygen species. *Am J Respir Cell Mol Biol,* 26**,** 447-52.

FLORES-VILLANUEVA, P. O., YUNIS, E. J., DELGADO, J. C., VITTINGHOFF, E., BUCHBINDER, S., LEUNG, J. Y., UGLIALORO, A. M., CLAVIJO, O. P., ROSENBERG, E. S., KALAMS, S. A., BRAUN, J. D., BOSWELL, S. L., WALKER, B. D. & GOLDFELD, A. E. (2001) Control of HIV-1 viremia and protection from AIDS are associated with HLA-Bw4 homozygosity. *Proc Natl Acad Sci U S A,* 98**,** 5140-5.

FONTENOT, J. D., RASMUSSEN, J. P., GAVIN, M. A. & RUDENSKY, A. Y. (2005) A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol,* 6**,** 1142-51.

FONTENOT, J. D. & RUDENSKY, A. Y. (2004) Molecular aspects of regulatory T cell development. *Semin Immunol,* 16**,** 73-80.

FORSSLUND, H., MIKKO, M., KARIMI, R., GRUNEWALD, J., WHEELOCK, A. M., WAHLSTROM, J. & SKOLD, C. M. (2014) Distribution of T-cell subsets in BAL fluid of patients with mild to moderate COPD depends on current smoking status and not airway obstruction. *Chest,* 145**,** 711-22.

FORSTER, R., MATTIS, A. E., KREMMER, E., WOLF, E., BREM, G. & LIPP, M. (1996) A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell,* 87**,** 1037-47.

FRANCUS, T., ROMANO, P. M., MANZO, G., FONACIER, L., ARANGO, N. & SZABO, P. (1992) IL-1, IL-6, and PDGF mRNA expression in alveolar cells following stimulation with a tobacco-derived antigen. *Cell Immunol,* 145**,** 156-74.

FRANKENBERGER, M., EDER, C., HOFER, T. P., HEIMBECK, I., SKOKANN, K., KASSNER, G., WEBER, N., MOLLER, W. & ZIEGLER-HEITBROCK, L. (2010) Chemokine expression by small sputum macrophages in COPD. *Mol Med,* 17**,** 762-70.

FRANKENBERGER, M., MENZEL, M., BETZ, R., KASSNER, G., WEBER, N., KOHLHAUFL, M., HAUSSINGER, K. & ZIEGLER-HEITBROCK, L. (2004) Characterization of a population of small macrophages in induced sputum of patients with chronic obstructive pulmonary disease and healthy volunteers. *Clin Exp Immunol,* 138**,** 507-16.

FREEMAN, C. M., HAN, M. K., MARTINEZ, F. J., MURRAY, S., LIU, L. X., CHENSUE, S. W., POLAK, T. J., SONSTEIN, J., TODT, J. C., AMES, T. M., ARENBERG, D. A., MELDRUM, C. A., GETTY, C., MCCLOSKEY, L. & CURTIS, J. L. (2010) Cytotoxic potential of lung CD8(+) T cells increases with chronic obstructive pulmonary disease severity and with in vitro stimulation by IL-18 or IL-15. *J Immunol,* 184**,** 6504-13.

FREEMAN, C. M., MARTINEZ, F. J., HAN, M. K., WASHKO, G. R., JR., MCCUBBREY, A. L., CHENSUE, S. W., ARENBERG, D. A., MELDRUM, C. A., MCCLOSKEY, L. & CURTIS, J. L. (2013) Lung

CD8+ T cells in COPD have increased expression of bacterial TLRs. *Respir Res,* 14**,** 13.

FREY, M., PACKIANATHAN, N. B., FEHNIGER, T. A., ROSS, M. E., WANG, W. C., STEWART, C. C., CALIGIURI, M. A. & EVANS, S. S. (1998) Differential expression and function of L-selectin on CD56bright and CD56dim natural killer cell subsets. *J Immunol,* 161**,** 400-8.

FROELICH, C. J., ORTH, K., TURBOV, J., SETH, P., GOTTLIEB, R., BABIOR, B., SHAH, G. M., BLEACKLEY, R. C., DIXIT, V. M. & HANNA, W. (1996) New paradigm for lymphocyte granule-mediated cytotoxicity. Target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. *J Biol Chem,* 271**,** 29073-9.

FUJII, H., NAKAGAWA, Y., SCHINDLER, U., KAWAHARA, A., MORI, H., GOUILLEUX, F., GRONER, B., IHLE, J. N., MINAMI, Y., MIYAZAKI, T. & ET AL. (1995) Activation of Stat5 by interleukin 2 requires a carboxyl-terminal region of the interleukin 2 receptor beta chain but is not essential for the proliferative signal transmission. *Proc Natl Acad Sci U S A,* 92**,** 5482-6.

FUJIMOTO, K., IMAIZUMI, T., YOSHIDA, H., TAKANASHI, S., OKUMURA, K. & SATOH, K. (2001) Interferon-gamma stimulates fractalkine expression in human bronchial epithelial cells and regulates mononuclear cell adherence. *Am J Respir Cell Mol Biol,* 25**,** 233-8.

FUKUCHI, Y., NISHIMURA, M., ICHINOSE, M., ADACHI, M., NAGAI, A., KURIYAMA, T., TAKAHASHI, K., NISHIMURA, K., ISHIOKA, S., AIZAWA, H. & ZAHER, C. (2004) COPD in Japan: the Nippon COPD Epidemiology study. *Respirology,* 9**,** 458-65.

GALDSTON, M., MELNICK, E. L., GOLDRING, R. M., LEVYTSKA, V., CURASI, C. A. & DAVIS, A. L. (1977) Interactions of neutrophil elastase, serum trypsin inhibitory activity, and smoking history as risk factors for chronic obstructive pulmonary disease in patients with MM, MZ, and ZZ phenotypes for alpha-antitrypsin. *Am Rev Respir Dis,* 116**,** 837-46.

GALLI, S. J., KALESNIKOFF, J., GRIMBALDESTON, M. A., PILIPONSKY, A. M., WILLIAMS, C. M. & TSAI, M. (2005) Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu Rev Immunol,* 23**,** 749-86.

GANESAN, S., UNGER, B. L., COMSTOCK, A. T., ANGEL, K. A., MANCUSO, P., MARTINEZ, F. J. & SAJJAN, U. S. (2012) Aberrantly activated EGFR contributes to enhanced IL-8 expression in COPD airways epithelial cells via regulation of nuclear FoxO3A. *Thorax,* 68**,** 131-41.

GARCIA-SANZ, J. A., MACDONALD, H. R., JENNE, D. E., TSCHOPP, J. & NABHOLZ, M. (1990) Cell specificity of granzyme gene expression. *J Immunol,* 145**,** 3111-8.

GARSON, D., DOKHELAR, M. C., WAKASUGI, H., MISHAL, Z. & TURSZ, T. (1985) HLA class-I and class-II antigen expression by human leukemic K562 cells and by Burkitt-K562 hybrids: modulation by differentiation inducers and interferon. *Exp Hematol,* 13**,** 885-90.

GASCHLER, G. J., ZAVITZ, C. C., BAUER, C. M., SKRTIC, M., LINDAHL, M., ROBBINS, C. S., CHEN, B. & STAMPFLI, M. R. (2008) Cigarette smoke exposure attenuates cytokine production by mouse alveolar macrophages. *Am J Respir Cell Mol Biol,* 38**,** 218-26.

GEE, K., GUZZO, C., CHE MAT, N. F., MA, W. & KUMAR, A. (2009) The IL-12 family of cytokines in infection, inflammation and autoimmune disorders. *Inflamm Allergy Drug Targets,* 8**,** 40-52.

GERBER, H. P., DIXIT, V. & FERRARA, N. (1998) Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J Biol Chem,* 273**,** 13313-6.

GERSHENFELD, H. K. & WEISSMAN, I. L. (1986) Cloning of a cDNA for a T cell-specific serine protease from a cytotoxic T lymphocyte. *Science,* 232**,** 854-8.

GIAID, A., YANAGISAWA, M., LANGLEBEN, D., MICHEL, R. P., LEVY, R., SHENNIB, H., KIMURA, S., MASAKI, T., DUGUID, W. P. & STEWART, D. J. (1993) Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N Engl J Med,* 328**,** 1732-9.

GIBSON, P. G., WOOLLEY, K. L., CARTY, K., MURREE-ALLEN, K. & SALTOS, N. (1998) Induced sputum eosinophil cationic protein (ECP) measurement in asthma and chronic obstructive airway disease (COAD). *Clin Exp Allergy,* 28**,** 1081-8.

GIRI, J. G., KUMAKI, S., AHDIEH, M., FRIEND, D. J., LOOMIS, A., SHANEBECK, K., DUBOSE, R., COSMAN, D., PARK, L. S. & ANDERSON, D. M. (1995) Identification and cloning of a novel IL-15 binding protein that is structurally related to the alpha chain of the IL-2 receptor. *Embo J,* 14**,** 3654-63.

GLADER, P., MOLLER, S., LILJA, J., WIESLANDER, E., LOFDAHL, C. G. & VON WACHENFELDT, K. (2006a) Cigarette smoke extract modulates respiratory defence mechanisms through effects on T-cells and airway epithelial cells. *Respir Med,* 100**,** 818-27.

GLADER, P., VON WACHENFELDT, K. & LOFDAHL, C. G. (2006b) Systemic CD4+ T-cell activation is correlated with FEV1 in smokers. *Respir Med,* 100**,** 1088-93.

GLIENKE, J., SOBANOV, Y., BROSTJAN, C., STEFFENS, C., NGUYEN, C., LEHRACH, H., HOFER, E. & FRANCIS, F. (1998) The genomic organization of NKG2C, E, F, and D receptor genes in the human natural killer gene complex. *Immunogenetics,* 48**,** 163-73.

GOMPERTZ, S., O'BRIEN, C., BAYLEY, D. L., HILL, S. L. & STOCKLEY, R. A. (2001) Changes in bronchial inflammation during acute exacerbations of chronic bronchitis. *Eur Respir J,* 17**,** 1112-9.

GONDEK, D. C., LU, L. F., QUEZADA, S. A., SAKAGUCHI, S. & NOELLE, R. J. (2005) Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol,* 174**,** 1783-6.

GOODRIDGE, J. P., WITT, C. S., CHRISTIANSEN, F. T. & WARREN, H. S. (2003) KIR2DL4 (CD158d) genotype influences expression and function in NK cells. *J Immunol,* 171**,** 1768-74.

GOSKER, H. R., WOUTERS, E. F., VAN DER VUSSE, G. J. & SCHOLS, A. M. (2000) Skeletal muscle dysfunction in chronic obstructive

pulmonary disease and chronic heart failure: underlying mechanisms and therapy perspectives. *Am J Clin Nutr,* 71**,** 1033-47.

GOSMAN, M. M., POSTMA, D. S., VONK, J. M., RUTGERS, B., LODEWIJK, M., SMITH, M., LUINGE, M. A., TEN HACKEN, N. H. & TIMENS, W. (2008) Association of mast cells with lung function in chronic obstructive pulmonary disease. *Respir Res,* 9**,** 64.

GOSSELINK, J. V., HAYASHI, S., ELLIOTT, W. M., XING, L., CHAN, B., YANG, L., WRIGHT, C., SIN, D., PARE, P. D., PIERCE, J. A., PIERCE, R. A., PATTERSON, A., COOPER, J. & HOGG, J. C. (2010) Differential expression of tissue repair genes in the pathogenesis of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 181**,** 1329-35.

GRABSTEIN, K. H., EISENMAN, J., SHANEBECK, K., RAUCH, C., SRINIVASAN, S., FUNG, V., BEERS, C., RICHARDSON, J., SCHOENBORN, M. A., AHDIEH, M. & ET AL. (1994) Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science,* 264**,** 965-8.

GREGOIRE, C., CHASSON, L., LUCI, C., TOMASELLO, E., GEISSMANN, F., VIVIER, E. & WALZER, T. (2007) The trafficking of natural killer cells. *Immunol Rev,* 220**,** 169-82.

GROSSMAN, W. J., VERBSKY, J. W., TOLLEFSEN, B. L., KEMPER, C., ATKINSON, J. P. & LEY, T. J. (2004) Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood,* 104**,** 2840-8.

GRUBEK-JAWORSKA, H., PAPLINSKA, M., HERMANOWICZ-SALAMON, J., BIALEK-GOSK, K., DABROWSKA, M., GRABCZAK, E., DOMAGALA-KULAWIK, J., STEPIEN, J. & CHAZAN, R. (2012) IL-6 and IL-13 in induced sputum of COPD and asthma patients: correlation with respiratory tests. *Respiration,* 84**,** 101-7.

GRUBOR-BAUK, B., SIMMONS, A., MAYRHOFER, G. & SPECK, P. G. (2003) Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V alpha 14-J alpha 281 TCR. *J Immunol,* 170**,** 1430-4.

GRUMELLI, S., CORRY, D. B., SONG, L. Z., SONG, L., GREEN, L., HUH, J., HACKEN, J., ESPADA, R., BAG, R., LEWIS, D. E. & KHERADMAND, F. (2004) An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema. *PLoS Med,* 1**,** e8.

GUDMUNDSSON, G., GISLASON, T., JANSON, C., LINDBERG, E., HALLIN, R., ULRIK, C. S., BRONDUM, E., NIEMINEN, M. M., AINE, T. & BAKKE, P. (2005) Risk factors for rehospitalisation in COPD: role of health status, anxiety and depression. *Eur Respir J,* 26**,** 414-9.

GUEDERS, M. M., FOIDART, J. M., NOEL, A. & CATALDO, D. D. (2006) Matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs in the respiratory tract: potential implications in asthma and other lung diseases. *Eur J Pharmacol,* 533**,** 133-44.

GUMPERZ, J. E., BARBER, L. D., VALIANTE, N. M., PERCIVAL, L., PHILLIPS, J. H., LANIER, L. L. & PARHAM, P. (1997) Conserved

and variable residues within the Bw4 motif of HLA-B make separable contributions to recognition by the NKB1 killer cell-inhibitory receptor. *J Immunol,* 158**,** 5237-41.

GUMPERZ, J. E., LITWIN, V., PHILLIPS, J. H., LANIER, L. L. & PARHAM, P. (1995) The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med,* 181**,** 1133-44.

GUMPERZ, J. E., MIYAKE, S., YAMAMURA, T. & BRENNER, M. B. (2002) Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med,* 195**,** 625-36.

GUSTAFSSON, A., ASMAN, B. & BERGSTROM, K. (2000) Cigarette smoking as an aggravating factor in inflammatory tissue-destructive diseases. Increase in tumor necrosis Factor-alpha priming of peripheral neutrophils measured as generation of oxygen radicals. *Int J Clin Lab Res,* 30**,** 187-90.

GUTIERREZ-RAMOS, J. C., LLOYD, C., KAPSENBERG, M. L., GONZALO, J. A. & COYLE, A. J. (2000) Non-redundant functional groups of chemokines operate in a coordinate manner during the inflammatory response in the lung. *Immunol Rev,* 177**,** 31-42.

HACKER, H. & KARIN, M. (2006) Regulation and function of IKK and IKK-related kinases. *Sci STKE,* 2006**,** re13.

HAGEMAN, G. J., LARIK, I., PENNINGS, H. J., HAENEN, G. R., WOUTERS, E. F. & BAST, A. (2003) Systemic poly(ADP-ribose) polymerase-1 activation, chronic inflammation, and oxidative stress in COPD patients. *Free Radic Biol Med,* 35**,** 140-8.

HALBERT, R. J., NATOLI, J. L., GANO, A., BADAMGARAV, E., BUIST, A. S. & MANNINO, D. M. (2006) Global burden of COPD: systematic review and meta-analysis. *Eur Respir J,* 28**,** 523-32.

HAMEED, A., LOWREY, D. M., LICHTENHELD, M. & PODACK, E. R. (1988) Characterization of three serine esterases isolated from human IL-2 activated killer cells. *J Immunol,* 141**,** 3142-7.

HAMILTON, J. A. (2002) GM-CSF in inflammation and autoimmunity. *Trends Immunol,* 23**,** 403-8.

HAMMOND, M. E., LAPOINTE, G. R., FEUCHT, P. H., HILT, S., GALLEGOS, C. A., GORDON, C. A., GIEDLIN, M. A., MULLENBACH, G. & TEKAMP-OLSON, P. (1995) IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors. *J Immunol,* 155**,** 1428-33.

HANSEN, D. S. & SCHOFIELD, L. (2004) Regulation of immunity and pathogenesis in infectious diseases by CD1d-restricted NKT cells. *Int J Parasitol,* 34**,** 15-25.

HARDAKER, E. L., BACON, A. M., CARLSON, K., ROSHAK, A. K., FOLEY, J. J., SCHMIDT, D. B., BUCKLEY, P. T., COMEGYS, M., PANETTIERI, R. A., JR., SARAU, H. M. & BELMONTE, K. E. (2004) Regulation of TNF-alpha- and IFN-gamma-induced CXCL10 expression: participation of the airway smooth muscle in the pulmonary inflammatory response in chronic obstructive pulmonary disease. *Faseb J,* 18**,** 191-3.

HASLETT, C. (1999) Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am J Respir Crit Care Med,* 160**,** S5-11.

HAUTAMAKI, R. D., KOBAYASHI, D. K., SENIOR, R. M. & SHAPIRO, S. D. (1997) Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science,* 277**,** 2002-4.

HAYDAY, A. & TIGELAAR, R. (2003) Immunoregulation in the tissues by gammadelta T cells. *Nat Rev Immunol,* 3**,** 233-42.

HAYES, M. P., BERREBI, G. A. & HENKART, P. A. (1989) Induction of target cell DNA release by the cytotoxic T lymphocyte granule protease granzyme A. *J Exp Med,* 170**,** 933-46.

HENKART, P. A. (1985) Mechanism of lymphocyte-mediated cytotoxicity. *Annu Rev Immunol,* 3**,** 31-58.

HENKART, P. A. (1994) Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity,* 1**,** 343-6.

HENSON, P. M., VANDIVIER, R. W. & DOUGLAS, I. S. (2006) Cell death, remodeling, and repair in chronic obstructive pulmonary disease? *Proc Am Thorac Soc,* 3**,** 713-7.

HIGHAM, M. A., PRIDE, N. B., ALIKHAN, A. & MORRELL, N. W. (2000) Tumour necrosis factor-alpha gene promoter polymorphism in chronic obstructive pulmonary disease. *Eur Respir J,* 15**,** 281-4.

HILL, A. T., CAMPBELL, E. J., BAYLEY, D. L., HILL, S. L. & STOCKLEY, R. A. (1999) Evidence for excessive bronchial inflammation during an acute exacerbation of chronic obstructive pulmonary disease in patients with alpha(1)-antitrypsin deficiency (PiZ). *Am J Respir Crit Care Med,* 160**,** 1968-75.

HILL, A. T., CAMPBELL, E. J., HILL, S. L., BAYLEY, D. L. & STOCKLEY, R. A. (2000) Association between airway bacterial load and markers of airway inflammation in patients with stable chronic bronchitis. *Am J Med,* 109**,** 288-95.

HILL, K., GEIST, R., GOLDSTEIN, R. S. & LACASSE, Y. (2008) Anxiety and depression in end-stage COPD. *Eur Respir J,* 31**,** 667-77.

HO, Q. T. & KUO, C. J. (2007) Vascular endothelial growth factor: biology and therapeutic applications. *Int J Biochem Cell Biol,* 39**,** 1349-57.

HODGE, G., HOLMES, M., JERSMANN, H., REYNOLDS, P. N. & HODGE, S. (2013) The drug efflux pump Pgp1 in pro-inflammatory lymphocytes is a target for novel treatment strategies in COPD. *Respir Res,* 14**,** 63.

HODGE, G., MUKARO, V., HOLMES, M., REYNOLDS, P. N. & HODGE, S. (2012) Enhanced cytotoxic function of natural killer and natural killer T-like cells associated with decreased CD94 (Kp43) in the chronic obstructive pulmonary disease airway. *Respirology,* 18**,** 369-76.

HODGE, S., HODGE, G., AHERN, J., JERSMANN, H., HOLMES, M. & REYNOLDS, P. N. (2007) Smoking alters alveolar macrophage recognition and phagocytic ability: implications in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol,* 37**,** 748-55.

HODGE, S., HODGE, G., HOLMES, J. M. & REYNOLDS, P. N. (2005a) Apoptosis in COPD. *Current Respiratory Medicine Reviews,* 1**,** 33-41.

HODGE, S., HODGE, G., HOLMES, M. & REYNOLDS, P. N. (2005b) Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. *Eur Respir J,* 25**,** 447-54.

HODGE, S., HODGE, G., NAIRN, J., HOLMES, M. & REYNOLDS, P. N. (2006) Increased airway granzyme b and perforin in current and ex-smoking COPD subjects. *Copd,* 3**,** 179-87.

HODGE, S., HODGE, G., SCICCHITANO, R., REYNOLDS, P. N. & HOLMES, M. (2003a) Alveolar macrophages from subjects with chronic obstructive pulmonary disease are deficient in their ability to phagocytose apoptotic airway epithelial cells. *Immunol Cell Biol,* 81**,** 289-96.

HODGE, S. J., HODGE, G. L., REYNOLDS, P. N., SCICCHITANO, R. & HOLMES, M. (2003b) Increased production of TGF-beta and apoptosis of T lymphocytes isolated from peripheral blood in COPD. *Am J Physiol Lung Cell Mol Physiol,* 285**,** L492-9.

HOFFMANN, D. & WYNDER, E. L. (1986) Chemical constituents and bioactivity of tobacco smoke. *IARC Sci Publ***,** 145-65.

HOGAN, A. E., CORRIGAN, M. A., O'REILLY, V., GAOATSWE, G., O'CONNELL, J., DOHERTY, D. G., LYNCH, L. & O'SHEA, D. (2011) Cigarette smoke alters the invariant natural killer T cell function and may inhibit anti-tumor responses. *Clin Immunol,* 140**,** 229-35.

HOGG, J. C., CHU, F., UTOKAPARCH, S., WOODS, R., ELLIOTT, W. M., BUZATU, L., CHERNIACK, R. M., ROGERS, R. M., SCIURBA, F. C., COXSON, H. O. & PARE, P. D. (2004) The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med,* 350**,** 2645-53.

HOGG, J. C., MACKLEM, P. T. & THURLBECK, W. M. (1968) Site and nature of airway obstruction in chronic obstructive lung disease. *N Engl J Med,* 278**,** 1355-60.

HOGG, J. C. & WALKER, B. A. (1995) Polymorphonuclear leucocyte traffic in lung inflammation. *Thorax,* 50**,** 819-20.

HOGGER, P., DREIER, J., DROSTE, A., BUCK, F. & SORG, C. (1998) Identification of the integral membrane protein RM3/1 on human monocytes as a glucocorticoid-inducible member of the scavenger receptor cysteine-rich family (CD163). *J Immunol,* 161**,** 1883-90.

HOLT, P. G., CHALMER, J. E., ROBERTS, L. M., PAPADIMITRIOU, J. M., THOMAS, W. R. & KEAST, D. (1976) Low-tar and high-tar cigarettes. *Arch Environ Health,* 31**,** 258-65.

HOLTMEIER, W. & KABELITZ, D. (2005) gammadelta T cells link innate and adaptive immune responses. *Chem Immunol Allergy,* 86**,** 151-83.

HONDA, Y., TAKAHASHI, H., KUROKI, Y., AKINO, T. & ABE, S. (1996) Decreased contents of surfactant proteins A and D in BAL fluids of healthy smokers. *Chest,* 109**,** 1006-9.

HONG, S., SCHERER, D. C., SINGH, N., MENDIRATTA, S. K., SERIZAWA, I., KOEZUKA, Y. & VAN KAER, L. (1999) Lipid antigen presentation in the immune system: lessons learned from CD1d knockout mice. *Immunol Rev,* 169**,** 31-44.

HOU, J., SUN, Y., HAO, Y., ZHUO, J., LIU, X., BAI, P., HAN, J., ZHENG, X. & ZENG, H. (2013) Imbalance between subpopulations of regulatory T cells in COPD. *Thorax,* 68**,** 1131-9.

HOUCHINS, J. P., YABE, T., MCSHERRY, C. & BACH, F. H. (1991) DNA sequence analysis of NKG2, a family of related cDNA clones encoding

type II integral membrane proteins on human natural killer cells. *J Exp Med,* 173**,** 1017-20.

HUANG, N., LIU, L., WANG, X. Z., LIU, D., YIN, S. Y. & YANG, X. D. (2008) Association of interleukin (IL)-12 and IL-27 gene polymorphisms with chronic obstructive pulmonary disease in a Chinese population. *DNA Cell Biol,* 27**,** 527-31.

HUBER, A. R., KUNKEL, S. L., TODD, R. F., 3RD & WEISS, S. J. (1991) Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science,* 254**,** 99-102.

HULSMANN, A. R., RAATGEEP, H. R., SAXENA, P. R., KERREBIJN, K. F. & DE JONGSTE, J. C. (1994) Bradykinin-induced contraction of human peripheral airways mediated by both bradykinin beta 2 and thromboxane prostanoid receptors. *Am J Respir Crit Care Med,* 150**,** 1012-8.

ICHINOSE, M., SUGIURA, H., YAMAGATA, S., KOARAI, A. & SHIRATO, K. (2000) Increase in reactive nitrogen species production in chronic obstructive pulmonary disease airways. *Am J Respir Crit Care Med,* 162**,** 701-6.

ILUMETS, H., RYTILA, P., DEMEDTS, I., BRUSSELLE, G. G., SOVIJARVI, A., MYLLARNIEMI, M., SORSA, T. & KINNULA, V. L. (2007) Matrix metalloproteinases -8, -9 and -12 in smokers and patients with stage 0 COPD. *Int J Chron Obstruct Pulmon Dis,* 2**,** 369-79.

ILUMETS, H., RYTILA, P. H., SOVIJARVI, A. R., TERVAHARTIALA, T., MYLLARNIEMI, M., SORSA, T. A. & KINNULA, V. L. (2008) Transient elevation of neutrophil proteinases in induced sputum during COPD exacerbation. *Scand J Clin Lab Invest,* 68**,** 618-23.

IMAI, K., DALAL, S. S., CHEN, E. S., DOWNEY, R., SCHULMAN, L. L., GINSBURG, M. & D'ARMIENTO, J. (2001) Human collagenase (matrix metalloproteinase-1) expression in the lungs of patients with emphysema. *Am J Respir Crit Care Med,* 163**,** 786-91.

IMAI, K., MERCER, B. A., SCHULMAN, L. L., SONETT, J. R. & D'ARMIENTO, J. M. (2005) Correlation of lung surface area to apoptosis and proliferation in human emphysema. *Eur Respir J,* 25**,** 250-8.

IMAIZUMI, T., ALBERTINE, K. H., JICHA, D. L., MCINTYRE, T. M., PRESCOTT, S. M. & ZIMMERMAN, G. A. (1997) Human endothelial cells synthesize ENA-78: relationship to IL-8 and to signaling of PMN adhesion. *Am J Respir Cell Mol Biol,* 17**,** 181-92.

IMAOKA, H., HOSHINO, T., TAKEI, S., KINOSHITA, T., OKAMOTO, M., KAWAYAMA, T., KATO, S., IWASAKI, H., WATANABE, K. & AIZAWA, H. (2008) Interleukin-18 production and pulmonary function in COPD. *Eur Respir J,* 31**,** 287-97.

INNES, A. L., WOODRUFF, P. G., FERRANDO, R. E., DONNELLY, S., DOLGANOV, G. M., LAZARUS, S. C. & FAHY, J. V. (2006) Epithelial mucin stores are increased in the large airways of smokers with airflow obstruction. *Chest,* 130**,** 1102-8.

IRANI, A. A., SCHECHTER, N. M., CRAIG, S. S., DEBLOIS, G. & SCHWARTZ, L. B. (1986) Two types of human mast cells that have

distinct neutral protease compositions. *Proc Natl Acad Sci U S A,* 83**,** 4464-8.

ISHII, T., MATSUSE, T., TERAMOTO, S., MATSUI, H., HOSOI, T., FUKUCHI, Y. & OUCHI, Y. (2000) Association between alpha-1-antichymotrypsin polymorphism and susceptibility to chronic obstructive pulmonary disease. *Eur J Clin Invest,* 30**,** 543-8.

JAMESON, J., WITHERDEN, D. & HAVRAN, W. L. (2003) T-cell effector mechanisms: gammadelta and CD1d-restricted subsets. *Curr Opin Immunol,* 15**,** 349-53.

JAMIESON, A. M., DIEFENBACH, A., MCMAHON, C. W., XIONG, N., CARLYLE, J. R. & RAULET, D. H. (2002) The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity,* 17**,** 19-29.

JANOFF, A., RAJU, L. & DEARING, R. (1983) Levels of elastase activity in bronchoalveolar lavage fluids of healthy smokers and nonsmokers. *Am Rev Respir Dis,* 127**,** 540-4.

JANSSON, S. A., ANDERSSON, F., BORG, S., ERICSSON, A., JONSSON, E. & LUNDBACK, B. (2002) Costs of COPD in Sweden according to disease severity. *Chest,* 122**,** 1994-2002.

JEWETT, A. & TSENG, H. C. (2013) Tumor induced inactivation of natural killer cell cytotoxic function; implication in growth, expansion and differentiation of cancer stem cells. *J Cancer,* 2**,** 443-57.

JIANG, H. & CHESS, L. (2006) Regulation of immune responses by T cells. *N Engl J Med,* 354**,** 1166-76.

JOHNSTON, S. L., PAPI, A., BATES, P. J., MASTRONARDE, J. G., MONICK, M. M. & HUNNINGHAKE, G. W. (1998) Low grade rhinovirus infection induces a prolonged release of IL-8 in pulmonary epithelium. *J Immunol,* 160**,** 6172-81.

JONES, C. E. & CHAN, K. (2002) Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells. *Am J Respir Cell Mol Biol,* 26**,** 748-53.

JONULEIT, H., WIEDEMANN, K., MULLER, G., DEGWERT, J., HOPPE, U., KNOP, J. & ENK, A. H. (1997) Induction of IL-15 messenger RNA and protein in human blood-derived dendritic cells: a role for IL-15 in attraction of T cells. *J Immunol,* 158**,** 2610-5.

JOOSTEN, L. A., HEINHUIS, B., NETEA, M. G. & DINARELLO, C. A. (2013) Novel insights into the biology of interleukin-32. *Cell Mol Life Sci*.

KAKIMI, K., GUIDOTTI, L. G., KOEZUKA, Y. & CHISARI, F. V. (2000) Natural killer T cell activation inhibits hepatitis B virus replication in vivo. *J Exp Med,* 192**,** 921-30.

KALENDERIAN, R., RAJU, L., ROTH, W., SCHWARTZ, L. B., GRUBER, B. & JANOFF, A. (1988) Elevated histamine and tryptase levels in smokers' bronchoalveolar lavage fluid. Do lung mast cells contribute to smokers' emphysema? *Chest,* 94**,** 119-23.

KANAZAWA, H., SHIRAISHI, S., HIRATA, K. & YOSHIKAWA, J. (2003) Imbalance between levels of nitrogen oxides and peroxynitrite inhibitory activity in chronic obstructive pulmonary disease. *Thorax,* 58**,** 106-9.

KANAZAWA, H. & YOSHIKAWA, J. (2005) Elevated oxidative stress and reciprocal reduction of vascular endothelial growth factor levels with severity of COPD. *Chest,* 128**,** 3191-7.

KANEGANE, H. & TOSATO, G. (1996) Activation of naive and memory T cells by interleukin-15. *Blood,* 88**,** 230-5.

KARIMI, M., CAO, T. M., BAKER, J. A., VERNERIS, M. R., SOARES, L. & NEGRIN, R. S. (2005) Silencing human NKG2D, DAP10, and DAP12 reduces cytotoxicity of activated CD8+ T cells and NK cells. *J Immunol,* 175**,** 7819-28.

KAUR, M., SMYTH, L. J., CADDEN, P., GRUNDY, S., RAY, D., PLUMB, J. & SINGH, D. (2012) T lymphocyte insensitivity to corticosteroids in chronic obstructive pulmonary disease. *Respir Res,* 13**,** 20.

KAWAHARA, A., MINAMI, Y., MIYAZAKI, T., IHLE, J. N. & TANIGUCHI, T. (1995) Critical role of the interleukin 2 (IL-2) receptor gamma-chain-associated Jak3 in the IL-2-induced c-fos and c-myc, but not bcl-2, gene induction. *Proc Natl Acad Sci U S A,* 92**,** 8724-8.

KAWASAKI, Y., SAITO, T., SHIROTA-SOMEYA, Y., IKEGAMI, Y., KOMANO, H., LEE, M. H., FROELICH, C. J., SHINOHARA, N. & TAKAYAMA, H. (2000) Cell death-associated translocation of plasma membrane components induced by CTL. *J Immunol,* 164**,** 4641-8.

KEATINGS, V. M. & BARNES, P. J. (1997) Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. *Am J Respir Crit Care Med,* 155**,** 449-53.

KEATINGS, V. M., COLLINS, P. D., SCOTT, D. M. & BARNES, P. J. (1996) Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med,* 153**,** 530-4.

KENNA, T., GOLDEN-MASON, L., PORCELLI, S. A., KOEZUKA, Y., HEGARTY, J. E., O'FARRELLY, C. & DOHERTY, D. G. (2003) NKT cells from normal and tumor-bearing human livers are phenotypically and functionally distinct from murine NKT cells. *J Immunol,* 171**,** 1775-9.

KHARITONOV, S. A., ROBBINS, R. A., YATES, D., KEATINGS, V. & BARNES, P. J. (1995) Acute and chronic effects of cigarette smoking on exhaled nitric oxide. *Am J Respir Crit Care Med,* 152**,** 609-12.

KIKUCHI, A., NIEDA, M., SCHMIDT, C., KOEZUKA, Y., ISHIHARA, S., ISHIKAWA, Y., TADOKORO, K., DURRANT, S., BOYD, A., JUJI, T. & NICOL, A. (2001) In vitro anti-tumour activity of alpha-galactosylceramide-stimulated human invariant Valpha24+NKT cells against melanoma. *Br J Cancer,* 85**,** 741-6.

KIM, C. H., BUTCHER, E. C. & JOHNSTON, B. (2002) Distinct subsets of human Valpha24-invariant NKT cells: cytokine responses and chemokine receptor expression. *Trends Immunol,* 23**,** 516-9.

KIM, C. H., ROTT, L. S., CLARK-LEWIS, I., CAMPBELL, D. J., WU, L. & BUTCHER, E. C. (2001) Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med,* 193**,** 1373-81.

KIM, E. Y., BATTAILE, J. T., PATEL, A. C., YOU, Y., AGAPOV, E., GRAYSON, M. H., BENOIT, L. A., BYERS, D. E., ALEVY, Y., TUCKER, J., SWANSON, S., TIDWELL, R., TYNER, J. W., MORTON, J. D., CASTRO, M., POLINENI, D., PATTERSON, G. A., SCHWENDENER, R. A., ALLARD, J. D., PELTZ, G. & HOLTZMAN, M. J. (2008) Persistent activation of an innate immune response translates respiratory viral infection into chronic lung disease. *Nat Med,* 14**,** 633-40.

KIM, H. Y., KIM, H. J., MIN, H. S., KIM, S., PARK, W. S., PARK, S. H. & CHUNG, D. H. (2005) NKT cells promote antibody-induced joint inflammation by suppressing transforming growth factor beta1 production. *J Exp Med,* 201**,** 41-7.

KIM, W. D., EIDELMAN, D. H., IZQUIERDO, J. L., GHEZZO, H., SAETTA, M. P. & COSIO, M. G. (1991) Centrilobular and panlobular emphysema in smokers. Two distinct morphologic and functional entities. *Am Rev Respir Dis,* 144**,** 1385-90.

KING, A. E., CRITCHLEY, H. O. & KELLY, R. W. (2003) Innate immune defences in the human endometrium. *Reprod Biol Endocrinol,* 1**,** 116.

KISHI, A., TAKAMORI, Y., OGAWA, K., TAKANO, S., TOMITA, S., TANIGAWA, M., NIMAN, M., KISHIDA, T. & FUJITA, S. (2002) Differential expression of granulysin and perforin by NK cells in cancer patients and correlation of impaired granulysin expression with progression of cancer. *Cancer Immunol Immunother,* 50**,** 604-14.

KOJIMA, H., SHINOHARA, N., HANAOKA, S., SOMEYA-SHIROTA, Y., TAKAGAKI, Y., OHNO, H., SAITO, T., KATAYAMA, T., YAGITA, H., OKUMURA, K. & ET AL. (1994) Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. *Immunity,* 1**,** 357-64.

KOJIMA, J., ARAYA, J., HARA, H., ITO, S., TAKASAKA, N., KOBAYASHI, K., FUJII, S., TSURUSHIGE, C., NUMATA, T., ISHIKAWA, T., SHIMIZU, K., KAWAISHI, M., SAITO, K., KAMIYA, N., HIRANO, J., ODAKA, M., MORIKAWA, T., HANO, H., ARAI, S., MIYAZAKI, T., KANEKO, Y., NAKAYAMA, K. & KUWANO, K. (2013) Apoptosis inhibitor of macrophage (AIM) expression in alveolar macrophages in COPD. *Respir Res,* 14**,** 30.

KOLLERT, F., PROBST, C., MULLER-QUERNHEIM, J., ZISSEL, G. & PRASSE, A. (2009) CCL18 production is decreased in alveolar macrophages from cigarette smokers. *Inflammation,* 32**,** 163-8.

KONJEVIC, G., SCHLESINGER, B., CHENG, L., OLSEN, K. J., PODACK, E. R. & SPUZIC, I. (1995) Analysis of perforin expression in human peripheral blood lymphocytes, CD56+ natural killer cell subsets and its induction by interleukin-2. *Immunol Invest,* 24**,** 499-507.

KRANENBURG, A. R., WILLEMS-WIDYASTUTI, A., MOOI, W. J., SAXENA, P. R., STERK, P. J., DE BOER, W. I. & SHARMA, H. S. (2005) Chronic obstructive pulmonary disease is associated with enhanced bronchial expression of FGF-1, FGF-2, and FGFR-1. *J Pathol,* 206**,** 28-38.

KRENSKY, A. M. & CLAYBERGER, C. (2005) Granulysin: a novel host defense molecule. *Am J Transplant,* 5**,** 1789-92.

KRONENBERG, M. & GAPIN, L. (2002) The unconventional lifestyle of NKT cells. *Nat Rev Immunol,* 2**,** 557-68.

KU, C. C., MURAKAMI, M., SAKAMOTO, A., KAPPLER, J. & MARRACK, P. (2000) Control of homeostasis of CD8+ memory T cells by opposing cytokines. *Science,* 288**,** 675-8.

KUMAR, V. & SHARMA, A. (2010) Mast cells: emerging sentinel innate immune cells with diverse role in immunity. *Mol Immunol,* 48**,** 14-25.

KUNZ, L. I., LAPPERRE, T. S., SNOECK-STROBAND, J. B., BUDULAC, S. E., TIMENS, W., VAN WIJNGAARDEN, S., SCHRUMPF, J. A., RABE, K. F., POSTMA, D. S., STERK, P. J. & HIEMSTRA, P. S. (2011) Smoking status and anti-inflammatory macrophages in bronchoalveolar lavage and induced sputum in COPD. *Respir Res,* 12**,** 34.

KUSCHNER, W. G., D'ALESSANDRO, A., WONG, H. & BLANC, P. D. (1996) Dose-dependent cigarette smoking-related inflammatory responses in healthy adults. *Eur Respir J,* 9**,** 1989-94.

KWON, O. J., AU, B. T., COLLINS, P. D., ADCOCK, I. M., MAK, J. C., ROBBINS, R. R., CHUNG, K. F. & BARNES, P. J. (1994) Tumor necrosis factor-induced interleukin-8 expression in cultured human airway epithelial cells. *Am J Physiol,* 267**,** L398-405.

LA MOTTE-MOHS, R. N., HERER, E. & ZUNIGA-PFLUCKER, J. C. (2005) Induction of T-cell development from human cord blood hematopoietic stem cells by Delta-like 1 in vitro. *Blood,* 105**,** 1431-9.

LACOSTE, J. Y., BOUSQUET, J., CHANEZ, P., VAN VYVE, T., SIMONY-LAFONTAINE, J., LEQUEU, N., VIC, P., ENANDER, I., GODARD, P. & MICHEL, F. B. (1993) Eosinophilic and neutrophilic inflammation in asthma, chronic bronchitis, and chronic obstructive pulmonary disease. *J Allergy Clin Immunol,* 92**,** 537-48.

LAMBRECHT, B. N. & HAMMAD, H. (2003) Taking our breath away: dendritic cells in the pathogenesis of asthma. *Nat Rev Immunol,* 3**,** 994-1003.

LAMS, B. E., SOUSA, A. R., REES, P. J. & LEE, T. H. (2000) Subepithelial immunopathology of the large airways in smokers with and without chronic obstructive pulmonary disease. *Eur Respir J,* 15**,** 512-6.

LANE, N., ROBINS, R. A., CORNE, J. & FAIRCLOUGH, L. (2010) Regulation in chronic obstructive pulmonary disease: the role of regulatory T-cells and Th17 cells. *Clin Sci (Lond),* 119**,** 75-86.

LANIER, L. L. (1998) NK cell receptors. *Annu Rev Immunol,* 16**,** 359-93.

LANIER, L. L. (2009) DAP10- and DAP12-associated receptors in innate immunity. *Immunol Rev,* 227**,** 150-60.

LANIER, L. L., CORLISS, B. & PHILLIPS, J. H. (1997) Arousal and inhibition of human NK cells. *Immunol Rev,* 155**,** 145-54.

LANIER, L. L., CORLISS, B., WU, J. & PHILLIPS, J. H. (1998) Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity,* 8**,** 693-701.

LANIER, L. L., LE, A. M., CIVIN, C. I., LOKEN, M. R. & PHILLIPS, J. H. (1986) The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J Immunol,* 136**,** 4480-6.

LANIER, L. L., TESTI, R., BINDL, J. & PHILLIPS, J. H. (1989) Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. *J Exp Med,* 169**,** 2233-8.

LAPIDOT, T., DAR, A. & KOLLET, O. (2005) How do stem cells find their way home? *Blood,* 106**,** 1901-10.

LAPPALAINEN, U., WHITSETT, J. A., WERT, S. E., TICHELAAR, J. W. & BRY, K. (2005) Interleukin-1beta causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung. *Am J Respir Cell Mol Biol,* 32**,** 311-8.

LAURITSEN, J. P., HAKS, M. C., LEFEBVRE, J. M., KAPPES, D. J. & WIEST, D. L. (2006) Recent insights into the signals that control alphabeta/gammadelta-lineage fate. *Immunol Rev,* 209**,** 176-90.

LAZETIC, S., CHANG, C., HOUCHINS, J. P., LANIER, L. L. & PHILLIPS, J. H. (1996) Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. *J Immunol,* 157**,** 4741-5.

LECKIE, M. J., JENKINS, G. R., KHAN, J., SMITH, S. J., WALKER, C., BARNES, P. J. & HANSEL, T. T. (2003) Sputum T lymphocytes in asthma, COPD and healthy subjects have the phenotype of activated intraepithelial T cells (CD69+ CD103+). *Thorax,* 58**,** 23-9.

LEE, B., RATAJCZAK, J., DOMS, R. W., GEWIRTZ, A. M. & RATAJCZAK, M. Z. (1999) Coreceptor/chemokine receptor expression on human hematopoietic cells: biological implications for human immunodeficiency virus-type 1 infection. *Blood,* 93**,** 1145-56.

LEE, C. W., LIN, C. C., LIN, W. N., LIANG, K. C., LUO, S. F., WU, C. B., WANG, S. W. & YANG, C. M. (2007a) TNF-alpha induces MMP-9 expression via activation of Src/EGFR, PDGFR/PI3K/Akt cascade and promotion of NF-kappaB/p300 binding in human tracheal smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol,* 292**,** L799-812.

LEE, J. C., LEE, K. M., KIM, D. W. & HEO, D. S. (2004) Elevated TGF-beta1 secretion and down-modulation of NKG2D underlies impaired NK cytotoxicity in cancer patients. *J Immunol,* 172**,** 7335-40.

LEE, P. T., BENLAGHA, K., TEYTON, L. & BENDELAC, A. (2002) Distinct functional lineages of human V(alpha)24 natural killer T cells. *J Exp Med,* 195**,** 637-41.

LEE, S. Y., KIM, M. J., KANG, H. G., YOO, S. S., CHOI, Y. Y., LEE, W. K., CHA, S. I., KIM, C. H., JUNG, T. H. & PARK, J. Y. (2010) Polymorphisms in matrix metalloproteinase-1, -9 and -12 genes and the risk of chronic obstructive pulmonary disease in a Korean population. *Respiration,* 80**,** 133-8.

LEE, W. K., RAMANATHAN, M., JR., SPANNHAKE, E. W. & LANE, A. P. (2007b) The cigarette smoke component acrolein inhibits expression of the innate immune components IL-8 and human beta-defensin 2 by sinonasal epithelial cells. *Am J Rhinol,* 21**,** 658-63.

LEITHAUSER, F., DHEIN, J., MECHTERSHEIMER, G., KORETZ, K., BRUDERLEIN, S., HENNE, C., SCHMIDT, A., DEBATIN, K. M., KRAMMER, P. H. & MOLLER, P. (1993) Constitutive and induced expression of APO-1, a new member of the nerve growth factor/tumor necrosis factor receptor superfamily, in normal and neoplastic cells. *Lab Invest,* 69**,** 415-29.

LENARDO, M. J. (1991) Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. *Nature,* 353**,** 858-61.

LENARDO, M. J. (1996) Fas and the art of lymphocyte maintenance. *J Exp Med,* 183**,** 721-4.

LEPIN, E. J., BASTIN, J. M., ALLAN, D. S., RONCADOR, G., BRAUD, V. M., MASON, D. Y., VAN DER MERWE, P. A., MCMICHAEL, A. J., BELL, J. I., POWIS, S. H. & O'CALLAGHAN, C. A. (2000) Functional characterization of HLA-F and binding of HLA-F tetramers to ILT2 and ILT4 receptors. *Eur J Immunol,* 30**,** 3552-61.

LEVY, E. M., ROBERTI, M. P. & MORDOH, J. (2013) Natural killer cells in human cancer: from biological functions to clinical applications. *J Biomed Biotechnol,* 2011**,** 676198.

LIM, S., ROCHE, N., OLIVER, B. G., MATTOS, W., BARNES, P. J. & CHUNG, K. F. (2000) Balance of matrix metalloprotease-9 and tissue inhibitor of metalloprotease-1 from alveolar macrophages in cigarette smokers. Regulation by interleukin-10. *Am J Respir Crit Care Med,* 162**,** 1355-60.

LIN, H., NIEDA, M., ROZENKOV, V. & NICOL, A. J. (2006) Analysis of the effect of different NKT cell subpopulations on the activation of CD4 and CD8 T cells, NK cells, and B cells. *Exp Hematol,* 34**,** 289-95.

LIN, J. X. & LEONARD, W. J. (1997) Signaling from the IL-2 receptor to the nucleus. *Cytokine Growth Factor Rev,* 8**,** 313-32.

LIN, J. X., MIGONE, T. S., TSANG, M., FRIEDMANN, M., WEATHERBEE, J. A., ZHOU, L., YAMAUCHI, A., BLOOM, E. T., MIETZ, J., JOHN, S. & ET AL. (1995) The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity,* 2**,** 331-9.

LINDEN, M., RASMUSSEN, J. B., PIITULAINEN, E., TUNEK, A., LARSON, M., TEGNER, H., VENGE, P., LAITINEN, L. A. & BRATTSAND, R. (1993) Airway inflammation in smokers with nonobstructive and obstructive chronic bronchitis. *Am Rev Respir Dis,* 148**,** 1226-32.

LISBONNE, M., DIEM, S., DE CASTRO KELLER, A., LEFORT, J., ARAUJO, L. M., HACHEM, P., FOURNEAU, J. M., SIDOBRE, S., KRONENBERG, M., TANIGUCHI, M., VAN ENDERT, P., DY, M., ASKENASE, P., RUSSO, M., VARGAFTIG, B. B., HERBELIN, A. & LEITE-DE-MORAES, M. C. (2003) Cutting edge: invariant V alpha 14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. *J Immunol,* 171**,** 1637-41.

LIU, C. C., RAFII, S., GRANELLI-PIPERNO, A., TRAPANI, J. A. & YOUNG, J. D. (1989) Perforin and serine esterase gene expression in stimulated human T cells. Kinetics, mitogen requirements, and effects of cyclosporin A. *J Exp Med,* 170**,** 2105-18.

LIU, H., HU, B., XU, D. & LIEW, F. Y. (2003) CD4+CD25+ regulatory T cells cure murine colitis: the role of IL-10, TGF-beta, and CTLA4. *J Immunol,* 171**,** 5012-7.

LIU, W., PUTNAM, A. L., XU-YU, Z., SZOT, G. L., LEE, M. R., ZHU, S., GOTTLIEB, P. A., KAPRANOV, P., GINGERAS, T. R., FAZEKAS

DE ST GROTH, B., CLAYBERGER, C., SOPER, D. M., ZIEGLER, S. F. & BLUESTONE, J. A. (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med,* 203**,** 1701-11.

LIU, Y. J., SOUMELIS, V., WATANABE, N., ITO, T., WANG, Y. H., MALEFYT RDE, W., OMORI, M., ZHOU, B. & ZIEGLER, S. F. (2007) TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. *Annu Rev Immunol,* 25**,** 193-219.

LJUNGGREN, H. G. & KARRE, K. (1990) In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today,* 11**,** 237-44.

LLANO, M., LEE, N., NAVARRO, F., GARCIA, P., ALBAR, J. P., GERAGHTY, D. E. & LOPEZ-BOTET, M. (1998) HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. *Eur J Immunol,* 28**,** 2854-63.

LOCATI, M., BONECCHI, R. & CORSI, M. M. (2005) Chemokines and their receptors: roles in specific clinical conditions and measurement in the clinical laboratory. *Am J Clin Pathol,* 123 Suppl**,** S82-95.

LOHWASSER, S., HANDE, P., MAGER, D. L. & TAKEI, F. (1999) Cloning of murine NKG2A, B and C: second family of C-type lectin receptors on murine NK cells. *Eur J Immunol,* 29**,** 755-61.

LOTZOVA, E., SAVARY, C. A. & CHAMPLIN, R. E. (1993) Genesis of human oncolytic natural killer cells from primitive CD34+CD33- bone marrow progenitors. *J Immunol,* 150**,** 5263-9.

LOZA, M. J., PETERS, S. P., ZANGRILLI, J. G. & PERUSSIA, B. (2002) Distinction between IL-13+ and IFN-gamma+ natural killer cells and regulation of their pool size by IL-4. *Eur J Immunol,* 32**,** 413-23.

LYMAN, S. D. & JACOBSEN, S. E. (1998) c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. *Blood,* 91**,** 1101-34.

MAASHO, K., OPOKU-ANANE, J., MARUSINA, A. I., COLIGAN, J. E. & BORREGO, F. (2005) NKG2D is a costimulatory receptor for human naive CD8+ T cells. *J Immunol,* 174**,** 4480-4.

MACATONIA, S. E., HOSKEN, N. A., LITTON, M., VIEIRA, P., HSIEH, C. S., CULPEPPER, J. A., WYSOCKA, M., TRINCHIERI, G., MURPHY, K. M. & O'GARRA, A. (1995) Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol,* 154**,** 5071-9.

MACDONALD, H. R. (1995) NK1.1+ T cell receptor-alpha/beta+ cells: new clues to their origin, specificity, and function. *J Exp Med,* 182**,** 633-8.

MACNEE, W. (2001a) Oxidants/antioxidants and chronic obstructive pulmonary disease: pathogenesis to therapy. *Novartis Found Symp,* 234**,** 169-85; discussion 185-8.

MACNEE, W. (2001b) Oxidative stress and lung inflammation in airways disease. *Eur J Pharmacol,* 429**,** 195-207.

MAENO, T., HOUGHTON, A. M., QUINTERO, P. A., GRUMELLI, S., OWEN, C. A. & SHAPIRO, S. D. (2007) CD8+ T Cells are required for inflammation and destruction in cigarette smoke-induced emphysema in mice. *J Immunol,* 178**,** 8090-6.

MAESTRELLI, P., PASKA, C., SAETTA, M., TURATO, G., NOWICKI, Y., MONTI, S., FORMICHI, B., MINIATI, M. & FABBRI, L. M. (2003) Decreased haem oxygenase-1 and increased inducible nitric oxide synthase in the lung of severe COPD patients. *Eur Respir J,* 21**,** 971-6.

MAHER, J. & DAVIES, E. T. (2004) Targeting cytotoxic T lymphocytes for cancer immunotherapy. *Br J Cancer,* 91**,** 817-21.

MAJO, J., GHEZZO, H. & COSIO, M. G. (2001) Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema. *Eur Respir J,* 17**,** 946-53.

MAJOR, A. S., WILSON, M. T., MCCALEB, J. L., RU SU, Y., STANIC, A. K., JOYCE, S., VAN KAER, L., FAZIO, S. & LINTON, M. F. (2004) Quantitative and qualitative differences in proatherogenic NKT cells in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol,* 24**,** 2351-7.

MALOY, K. J. & POWRIE, F. (2005) Fueling regulation: IL-2 keeps CD4+ Treg cells fit. *Nat Immunol,* 6**,** 1071-2.

MALTA, F. M., BRUNO, F. R., CARVALHO, K. I., NASTRI, A. C., KALIL, J., CARRILHO, F. J., KALLAS, E. G. & PINHO, J. R. (2013) HCV viremia drives an increment of CD86 expression by myeloid dendritic cells. *J Med Virol,* 85**,** 1919-24.

MANEL, N., UNUTMAZ, D. & LITTMAN, D. R. (2008) The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol,* 9**,** 641-9.

MANNINO, D. M. & BUIST, A. S. (2007) Global burden of COPD: risk factors, prevalence, and future trends. *Lancet,* 370**,** 765-73.

MANTOVANI, B., RABINOVITCH, M. & NUSSENZWEIG, V. (1972) Phagocytosis of immune complexes by macrophages. Different roles of the macrophage receptor sites for complement (C3) and for immunoglobulin (IgG). *J Exp Med,* 135**,** 780-92.

MARKS-KONCZALIK, J., DUBOIS, S., LOSI, J. M., SABZEVARI, H., YAMADA, N., FEIGENBAUM, L., WALDMANN, T. A. & TAGAYA, Y. (2000) IL-2-induced activation-induced cell death is inhibited in IL-15 transgenic mice. *Proc Natl Acad Sci U S A,* 97**,** 11445-50.

MARQUES, L. J., TESCHLER, H., GUZMAN, J. & COSTABEL, U. (1997) Smoker's lung transplanted to a nonsmoker. Long-term detection of smoker's macrophages. *Am J Respir Crit Care Med,* 156**,** 1700-2.

MARQUEZ, M. E., MILLET, C., STEKMAN, H., CONESA, A., DEGLESNE, P. A., TORO, F., SANCTIS, J. D. & BLANCA, I. (2010) CD16 cross-linking induces increased expression of CD56 and production of IL-12 in peripheral NK cells. *Cell Immunol,* 264**,** 86-92.

MARTINEZ, F. O., HELMING, L. & GORDON, S. (2009) Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol,* 27**,** 451-83.

MAZIARZ, R. T., MENTZER, S. J., BURAKOFF, S. J. & FALLER, D. V. (1990) Distinct effects of interferon-gamma and MHC class I surface antigen levels on resistance of the K562 tumor cell line to natural killer-mediated lysis. *Cell Immunol,* 130**,** 329-38.

MCCUE, J. M., LINK, K. L., EATON, S. S. & FREED, B. M. (2000) Exposure to cigarette tar inhibits ribonucleotide reductase and blocks lymphocyte proliferation. *J Immunol,* 165**,** 6771-5.

MCDONALD, D. R., GOLDMAN, F., GOMEZ-DUARTE, O. D., ISSEKUTZ, A. C., KUMARARATNE, D. S., DOFFINGER, R. & GEHA, R. S. (2010) Impaired T-cell receptor activation in IL-1 receptor-associated kinase-4-deficient patients. *J Allergy Clin Immunol,* 126**,** 332-7, 337 e1-2.

MEJA, K. K., BARNES, P. J. & GIEMBYCZ, M. A. (1997) Characterization of the prostanoid receptor(s) on human blood monocytes at which prostaglandin E2 inhibits lipopolysaccharide-induced tumour necrosis factor-alpha generation. *Br J Pharmacol,* 122**,** 149-57.

MERCER, P. F., SHUTE, J. K., BHOWMIK, A., DONALDSON, G. C., WEDZICHA, J. A. & WARNER, J. A. (2005) MMP-9, TIMP-1 and inflammatory cells in sputum from COPD patients during exacerbation. *Respir Res,* 6**,** 151.

MESHI, B., VITALIS, T. Z., IONESCU, D., ELLIOTT, W. M., LIU, C., WANG, X. D., HAYASHI, S. & HOGG, J. C. (2002) Emphysematous lung destruction by cigarette smoke. The effects of latent adenoviral infection on the lung inflammatory response. *Am J Respir Cell Mol Biol,* 26**,** 52-7.

METCALFE, H. J., LEA, S., HUGHES, D., KHALAF, R., ABBOTT-BANNER, K. & SINGH, D. (2014) Effects of cigarette smoke on Toll-like receptor (TLR) activation of chronic obstructive pulmonary disease (COPD) macrophages. *Clin Exp Immunol,* 176**,** 461-72.

METKAR, S. S., MENAA, C., PARDO, J., WANG, B., WALLICH, R., FREUDENBERG, M., KIM, S., RAJA, S. M., SHI, L., SIMON, M. M. & FROELICH, C. J. (2008) Human and mouse granzyme A induce a proinflammatory cytokine response. *Immunity,* 29**,** 720-33.

METKAR, S. S., WANG, B., AGUILAR-SANTELISES, M., RAJA, S. M., UHLIN-HANSEN, L., PODACK, E., TRAPANI, J. A. & FROELICH, C. J. (2002) Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation. *Immunity,* 16**,** 417-28.

MIAN, M. F., LAUZON, N. M., STAMPFLI, M. R., MOSSMAN, K. L. & ASHKAR, A. A. (2008) Impairment of human NK cell cytotoxic activity and cytokine release by cigarette smoke. *J Leukoc Biol,* 83**,** 774-84.

MIAN, M. F., PEK, E. A., MOSSMAN, K. L., STAMPFLI, M. R. & ASHKAR, A. A. (2009) Exposure to cigarette smoke suppresses IL-15 generation and its regulatory NK cell functions in poly I:C-augmented human PBMCs. *Mol Immunol,* 46**,** 3108-16.

MINAMI, Y., KONO, T., MIYAZAKI, T. & TANIGUCHI, T. (1993) The IL-2 receptor complex: its structure, function, and target genes. *Annu Rev Immunol,* 11**,** 245-68.

MINGARI, M. C., PONTE, M., BERTONE, S., SCHIAVETTI, F., VITALE, C., BELLOMO, R., MORETTA, A. & MORETTA, L. (1998) HLA class I-specific inhibitory receptors in human T lymphocytes: interleukin 15-induced expression of CD94/NKG2A in superantigen-

or alloantigen-activated CD8+ T cells. *Proc Natl Acad Sci U S A,* 95**,** 1172-7.

MIO, T., ROMBERGER, D. J., THOMPSON, A. B., ROBBINS, R. A., HEIRES, A. & RENNARD, S. I. (1997) Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells. *Am J Respir Crit Care Med,* 155**,** 1770-6.

MIOTTO, D., RUGGIERI, M. P., BOSCHETTO, P., CAVALLESCO, G., PAPI, A., BONONI, I., PIOLA, C., MURER, B., FABBRI, L. M. & MAPP, C. E. (2003) Interleukin-13 and -4 expression in the central airways of smokers with chronic bronchitis. *Eur Respir J,* 22**,** 602-8.

MIYAZAKI, T., KAWAHARA, A., FUJII, H., NAKAGAWA, Y., MINAMI, Y., LIU, Z. J., OISHI, I., SILVENNOINEN, O., WITTHUHN, B. A., IHLE, J. N. & ET AL. (1994) Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. *Science,* 266**,** 1045-7.

MNASRIA, K., LAGARAINE, C., MANAA, J., LEBRANCHU, Y. & OUESLATI, R. (2008) Anti CD25 treatment of human dendritic cells modulates both their cytokine synthesis profiles and their capacity to activate allogeneic CD4 T cells: a potential tolerogenic effect. *Int Immunopharmacol,* 8**,** 414-22.

MOHAN, S. (2002a) Effects of cigarette smoke on the immune system. *Nat Rev Immunol,* 2**,** 372-377.

MOHAN, S. (2002b) Effects of cigarette smoke on the immune system. *Nat Rev Immunol,* 2**,** 372-377.

MOLET, S., BELLEGUIC, C., LENA, H., GERMAIN, N., BERTRAND, C. P., SHAPIRO, S. D., PLANQUOIS, J. M., DELAVAL, P. & LAGENTE, V. (2005) Increase in macrophage elastase (MMP-12) in lungs from patients with chronic obstructive pulmonary disease. *Inflamm Res,* 54**,** 31-6.

MONTANO, M., BECCERRIL, C., RUIZ, V., RAMOS, C., SANSORES, R. H. & GONZALEZ-AVILA, G. (2004) Matrix metalloproteinases activity in COPD associated with wood smoke. *Chest,* 125**,** 466-72.

MONTUSCHI, P., COLLINS, J. V., CIABATTONI, G., LAZZERI, N., CORRADI, M., KHARITONOV, S. A. & BARNES, P. J. (2000) Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with COPD and healthy smokers. *Am J Respir Crit Care Med,* 162**,** 1175-7.

MONTUSCHI, P., KHARITONOV, S. A., CIABATTONI, G. & BARNES, P. J. (2003) Exhaled leukotrienes and prostaglandins in COPD. *Thorax,* 58**,** 585-8.

MORETTA, A., BOTTINO, C., PENDE, D., TRIPODI, G., TAMBUSSI, G., VIALE, O., ORENGO, A., BARBARESI, M., MERLI, A., CICCONE, E. & ET AL. (1990) Identification of four subsets of human CD3-CD16+ natural killer (NK) cells by the expression of clonally distributed functional surface molecules: correlation between subset assignment of NK clones and ability to mediate specific alloantigen recognition. *J Exp Med,* 172**,** 1589-98.

MORGAN, D. A., RUSCETTI, F. W. & GALLO, R. (1976) Selective in vitro growth of T lymphocytes from normal human bone marrows. *Science,* 193**,** 1007-8.

MORISSETTE, M. C., PARENT, J. & MILOT, J. (2007) Perforin, granzyme B, and FasL expression by peripheral blood T lymphocytes in emphysema. *Respir Res,* 8**,** 62.

MORRISON, D., STRIETER, R. M., DONNELLY, S. C., BURDICK, M. D., KUNKEL, S. L. & MACNEE, W. (1998) Neutrophil chemokines in bronchoalveolar lavage fluid and leukocyte-conditioned medium from nonsmokers and smokers. *Eur Respir J,* 12**,** 1067-72.

MORTAZ, E., LAZAR, Z., KOENDERMAN, L., KRANEVELD, A. D., NIJKAMP, F. P. & FOLKERTS, G. (2009) Cigarette smoke attenuates the production of cytokines by human plasmacytoid dendritic cells and enhances the release of IL-8 in response to TLR-9 stimulation. *Respir Res,* 10**,** 47.

MOSMANN, T. R. & COFFMAN, R. L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol,* 7**,** 145-73.

MOSMANN, T. R., LI, L., HENGARTNER, H., KAGI, D., FU, W. & SAD, S. (1997) Differentiation and functions of T cell subsets. *Ciba Found Symp,* 204**,** 148-54; discussion 154-8.

MROZEK, E., ANDERSON, P. & CALIGIURI, M. A. (1996) Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. *Blood,* 87**,** 2632-40.

MUKAIDA, N. (2003) Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases. *Am J Physiol Lung Cell Mol Physiol,* 284**,** L566-77.

MURPHY, T. F. (2006) The role of bacteria in airway inflammation in exacerbations of chronic obstructive pulmonary disease. *Curr Opin Infect Dis,* 19**,** 225-30.

MURRAY, P. J. & WYNN, T. A. (2011) Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol,* 11**,** 723-37.

NADEL, J. A. (1991) Role of enzymes from inflammatory cells on airway submucosal gland secretion. *Respiration,* 58 Suppl 1**,** 3-5.

NAGAKI, M., SHIMURA, S., IROKAWA, T., SASAKI, T., OSHIRO, T., NARA, M., KAKUTA, Y. & SHIRATO, K. (1996) Bradykinin regulation of airway submucosal gland secretion: role of bradykinin receptor subtype. *Am J Physiol,* 270**,** L907-13.

NAGATA, S. & GOLSTEIN, P. (1995) The Fas death factor. *Science,* 267**,** 1449-56.

NAGLER, A., LANIER, L. L., CWIRLA, S. & PHILLIPS, J. H. (1989) Comparative studies of human FcRIII-positive and negative natural killer cells. *J Immunol,* 143**,** 3183-91.

NAKAI, Y., IWABUCHI, K., FUJII, S., ISHIMORI, N., DASHTSOODOL, N., WATANO, K., MISHIMA, T., IWABUCHI, C., TANAKA, S., BEZBRADICA, J. S., NAKAYAMA, T., TANIGUCHI, M., MIYAKE, S., YAMAMURA, T., KITABATAKE, A., JOYCE, S., VAN KAER, L. & ONOE, K. (2004) Natural killer T cells accelerate atherogenesis in mice. *Blood,* 104**,** 2051-9.

NAKAJIMA, H., CELLA, M., LANGEN, H., FRIEDLEIN, A. & COLONNA, M. (1999) Activating interactions in human NK cell recognition: the role of 2B4-CD48. *Eur J Immunol,* 29**,** 1676-83.

NAKAMURA, H., YOSHIMURA, K., JAFFE, H. A. & CRYSTAL, R. G. (1991) Interleukin-8 gene expression in human bronchial epithelial cells. *J Biol Chem,* 266**,** 19611-7.

NAKAMURA, H., YOSHIMURA, K., MCELVANEY, N. G. & CRYSTAL, R. G. (1992) Neutrophil elastase in respiratory epithelial lining fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line. *J Clin Invest,* 89**,** 1478-84.

NAKANISHI, K., YOSHIMOTO, T., TSUTSUI, H. & OKAMURA, H. (2001) Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol,* 19**,** 423-74.

NAMEKAWA, T., SNYDER, M. R., YEN, J. H., GOEHRING, B. E., LEIBSON, P. J., WEYAND, C. M. & GORONZY, J. J. (2000) Killer cell activating receptors function as costimulatory molecules on CD4+CD28null T cells clonally expanded in rheumatoid arthritis. *J Immunol,* 165**,** 1138-45.

NATHAN, C. (2002) Points of control in inflammation. *Nature,* 420**,** 846-52.

NATHAN, C. (2006) Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol,* 6**,** 173-82.

NICHOL, G., NIX, A., BARNES, P. J. & CHUNG, K. F. (1990) Prostaglandin F2 alpha enhancement of capsaicin induced cough in man: modulation by beta 2 adrenergic and anticholinergic drugs. *Thorax,* 45**,** 694-8.

NIESS, J. H., BRAND, S., GU, X., LANDSMAN, L., JUNG, S., MCCORMICK, B. A., VYAS, J. M., BOES, M., PLOEGH, H. L., FOX, J. G., LITTMAN, D. R. & REINECKER, H. C. (2005) CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science,* 307**,** 254-8.

NITTA, T., YAGITA, H., SATO, K. & OKUMURA, K. (1989) Involvement of CD56 (NKH-1/Leu-19 antigen) as an adhesion molecule in natural killer-target cell interaction. *J Exp Med,* 170**,** 1757-61.

NOCKER, R. E., SCHOONBROOD, D. F., VAN DE GRAAF, E. A., HACK, C. E., LUTTER, R., JANSEN, H. M. & OUT, T. A. (1996) Interleukin-8 in airway inflammation in patients with asthma and chronic obstructive pulmonary disease. *Int Arch Allergy Immunol,* 109**,** 183-91.

NOGUERA, A., BUSQUETS, X., SAULEDA, J., VILLAVERDE, J. M., MACNEE, W. & AGUSTI, A. G. (1998) Expression of adhesion molecules and G proteins in circulating neutrophils in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 158**,** 1664-8.

NOURI-SHIRAZI, M. & GUINET, E. (2003) Evidence for the immunosuppressive role of nicotine on human dendritic cell functions. *Immunology,* 109**,** 365-73.

NOURI-SHIRAZI, M. & GUINET, E. (2006) A possible mechanism linking cigarette smoke to higher incidence of respiratory infection and asthma. *Immunol Lett,* 103**,** 167-76.

O'SHAUGHNESSY, T. C., ANSARI, T. W., BARNES, N. C. & JEFFERY, P. K. (1997) Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. *Am J Respir Crit Care Med,* 155**,** 852-7.

ONG, K. C., EARNEST, A. & LU, S. J. (2005) A multidimensional grading system (BODE index) as predictor of hospitalization for COPD. *Chest,* 128**,** 3810-6.

ONG, K. C., LU, S. J. & SOH, C. S. (2006) Does the multidimensional grading system (BODE) correspond to differences in health status of patients with COPD? *Int J Chron Obstruct Pulmon Dis,* 1**,** 91-6.

OROZCO-LEVI, M., GARCIA-AYMERICH, J., VILLAR, J., RAMIREZ-SARMIENTO, A., ANTO, J. M. & GEA, J. (2006) Wood smoke exposure and risk of chronic obstructive pulmonary disease. *Eur Respir J,* 27**,** 542-6.

OUYANG, Y., VIRASCH, N., HAO, P., AUBREY, M. T., MUKERJEE, N., BIERER, B. E. & FREED, B. M. (2000) Suppression of human IL-1beta, IL-2, IFN-gamma, and TNF-alpha production by cigarette smoke extracts. *J Allergy Clin Immunol,* 106**,** 280-7.

OWEN, C. A., CAMPBELL, M. A., BOUKEDES, S. S. & CAMPBELL, E. J. (1997) Cytokines regulate membrane-bound leukocyte elastase on neutrophils: a novel mechanism for effector activity. *Am J Physiol,* 272**,** L385-93.

PAATS, M. S., BERGEN, I. M., HOOGSTEDEN, H. C., VAN DER EERDEN, M. M. & HENDRIKS, R. W. (2012) Systemic CD4+ and CD8+ T-cell cytokine profiles correlate with GOLD stage in stable COPD. *Eur Respir J,* 40**,** 330-7.

PABST, R. & TSCHERNIG, T. (1995) Lymphocytes in the lung: an often neglected cell. Numbers, characterization and compartmentalization. *Anat Embryol (Berl),* 192**,** 293-9.

PACHER, P., BECKMAN, J. S. & LIAUDET, L. (2007) Nitric oxide and peroxynitrite in health and disease. *Physiol Rev,* 87**,** 315-424.

PANZNER, P., LAFITTE, J. J., TSICOPOULOS, A., HAMID, Q. & TULIC, M. K. (2003) Marked up-regulation of T lymphocytes and expression of interleukin-9 in bronchial biopsies from patients With chronic bronchitis with obstruction. *Chest,* 124**,** 1909-15.

PAPI, A., BELLETTATO, C. M., BRACCIONI, F., ROMAGNOLI, M., CASOLARI, P., CARAMORI, G., FABBRI, L. M. & JOHNSTON, S. L. (2006a) Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. *Am J Respir Crit Care Med,* 173**,** 1114-21.

PAPI, A., CASONI, G., CARAMORI, G., GUZZINATI, I., BOSCHETTO, P., RAVENNA, F., CALIA, N., PETRUZZELLI, S., CORBETTA, L., CAVALLESCO, G., FORINI, E., SAETTA, M., CIACCIA, A. & FABBRI, L. M. (2004) COPD increases the risk of squamous histological subtype in smokers who develop non-small cell lung carcinoma. *Thorax,* 59**,** 679-81.

PAPI, A., LUPPI, F., FRANCO, F. & FABBRI, L. M. (2006b) Pathophysiology of exacerbations of chronic obstructive pulmonary disease. *Proc Am Thorac Soc,* 3**,** 245-51.

PAPI, A., ROMAGNOLI, M., BARALDO, S., BRACCIONI, F., GUZZINATI, I., SAETTA, M., CIACCIA, A. & FABBRI, L. M. (2000) Partial reversibility of airflow limitation and increased exhaled NO and sputum eosinophilia in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 162**,** 1773-7.

PARASA, V. R., SIKHAMANI, R. & RAJA, A. (2012) Effect of recombinant cytokines on the expression of natural killer cell receptors from patients with TB or/and HIV infection. *PLoS One,* 7**,** e37448.

PAREDI, P., KHARITONOV, S. A., LEAK, D., WARD, S., CRAMER, D. & BARNES, P. J. (2000) Exhaled ethane, a marker of lipid peroxidation, is elevated in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 162**,** 369-73.

PAREKH, V. V., SINGH, A. K., WILSON, M. T., OLIVARES-VILLAGOMEZ, D., BEZBRADICA, J. S., INAZAWA, H., EHARA, H., SAKAI, T., SERIZAWA, I., WU, L., WANG, C. R., JOYCE, S. & VAN KAER, L. (2004) Quantitative and qualitative differences in the in vivo response of NKT cells to distinct alpha- and beta-anomeric glycolipids. *J Immunol,* 173**,** 3693-706.

PARHAM, P. (2005) MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol,* 5**,** 201-14.

PARK, J. W., RYTER, S. W. & CHOI, A. M. (2007) Functional significance of apoptosis in chronic obstructive pulmonary disease. *Copd,* 4**,** 347-53.

PARKER, L. C., WHYTE, M. K., VOGEL, S. N., DOWER, S. K. & SABROE, I. (2004) Toll-like receptor (TLR)2 and TLR4 agonists regulate CCR expression in human monocytic cells. *J Immunol,* 172**,** 4977-86.

PARKIN, J. & COHEN, B. (2001) An overview of the immune system. *Lancet,* 357**,** 1777-89.

PAROLINI, S., BOTTINO, C., FALCO, M., AUGUGLIARO, R., GILIANI, S., FRANCESCHINI, R., OCHS, H. D., WOLF, H., BONNEFOY, J. Y., BIASSONI, R., MORETTA, L., NOTARANGELO, L. D. & MORETTA, A. (2000) X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells. *J Exp Med,* 192**,** 337-46.

PASTERNACK, M. S., VERRET, C. R., LIU, M. A. & EISEN, H. N. (1986) Serine esterase in cytolytic T lymphocytes. *Nature,* 322**,** 740-3.

PATEL, I. S., SEEMUNGAL, T. A., WILKS, M., LLOYD-OWEN, S. J., DONALDSON, G. C. & WEDZICHA, J. A. (2002) Relationship between bacterial colonisation and the frequency, character, and severity of COPD exacerbations. *Thorax,* 57**,** 759-64.

PATTERSON, S., CHAIDOS, A., NEVILLE, D. C., POGGI, A., BUTTERS, T. D., ROBERTS, I. A. & KARADIMITRIS, A. (2008) Human invariant NKT cells display alloreactivity instructed by invariant TCR-CD1d interaction and killer Ig receptors. *J Immunol,* 181**,** 3268-76.

PAVORD, I. D. & TATTERSFIELD, A. E. (1995) Bronchoprotective role for endogenous prostaglandin E2. *Lancet,* 345**,** 436-8.

PEINADO, V. I., BARBERA, J. A., ABATE, P., RAMIREZ, J., ROCA, J., SANTOS, S. & RODRIGUEZ-ROISIN, R. (1999) Inflammatory reaction in pulmonary muscular arteries of patients with mild chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 159**,** 1605-11.

PENDE, D., CASTRICONI, R., ROMAGNANI, P., SPAGGIARI, G. M., MARCENARO, S., DONDERO, A., LAZZERI, E., LASAGNI, L., MARTINI, S., RIVERA, P., CAPOBIANCO, A., MORETTA, L.,

MORETTA, A. & BOTTINO, C. (2006) Expression of the DNAM-1 ligands, Nectin-2 (CD112) and poliovirus receptor (CD155), on dendritic cells: relevance for natural killer-dendritic cell interaction. *Blood,* 107**,** 2030-6.

PERENO, R., GAGGERO, A., SCUDELETTI, M., LANZA, L., MEAZZA, R., MISHAL, Z., JASMIN, C., INDIVERI, F., FERRINI, S. & AZZARONE, B. (1999) IL-15/IL-15R alpha intracellular trafficking in human cells and protection from apoptosis. *Ann N Y Acad Sci,* 876**,** 236-45.

PERENO, R., GIRON-MICHEL, J., GAGGERO, A., CAZES, E., MEAZZA, R., MONETTI, M., MONACO, E., MISHAL, Z., JASMIN, C., INDIVERI, F., FERRINI, S. & AZZARONE, B. (2000) IL-15/IL-15Ralpha intracellular trafficking in human melanoma cells and signal transduction through the IL-15Ralpha. *Oncogene,* 19**,** 5153-62.

PESCI, A., BALBI, B., MAJORI, M., CACCIANI, G., BERTACCO, S., ALCIATO, P. & DONNER, C. F. (1998) Inflammatory cells and mediators in bronchial lavage of patients with chronic obstructive pulmonary disease. *Eur Respir J,* 12**,** 380-6.

PETERSEN, A. M., PENKOWA, M., IVERSEN, M., FRYDELUND-LARSEN, L., ANDERSEN, J. L., MORTENSEN, J., LANGE, P. & PEDERSEN, B. K. (2007) Elevated levels of IL-18 in plasma and skeletal muscle in chronic obstructive pulmonary disease. *Lung,* 185**,** 161-71.

PHILLIPS, J. H., GUMPERZ, J. E., PARHAM, P. & LANIER, L. L. (1995) Superantigen-dependent, cell-mediated cytotoxicity inhibited by MHC class I receptors on T lymphocytes. *Science,* 268**,** 403-5.

PIERROU, S., BROBERG, P., O'DONNELL, R. A., PAWLOWSKI, K., VIRTALA, R., LINDQVIST, E., RICHTER, A., WILSON, S. J., ANGCO, G., MOLLER, S., BERGSTRAND, H., KOOPMANN, W., WIESLANDER, E., STROMSTEDT, P. E., HOLGATE, S. T., DAVIES, D. E., LUND, J. & DJUKANOVIC, R. (2007) Expression of genes involved in oxidative stress responses in airway epithelial cells of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 175**,** 577-86.

PIGUET, P. F., GRAU, G., IRLE, C. & VASSALLI, P. (1986) Administration of recombinant interleukin 2 to mice enhances production of hemopoietic and natural killer cells. *Eur J Immunol,* 16**,** 1257-61.

PINAMONTI, S., LEIS, M., BARBIERI, A., LEONI, D., MUZZOLI, M., SOSTERO, S., CHICCA, M. C., CARRIERI, A., RAVENNA, F., FABBRI, L. M. & CIACCIA, A. (1998) Detection of xanthine oxidase activity products by EPR and HPLC in bronchoalveolar lavage fluid from patients with chronic obstructive pulmonary disease. *Free Radic Biol Med,* 25**,** 771-9.

PITSIOU, G., KYRIAZIS, G., HATZIZISI, O., ARGYROPOULOU, P., MAVROFRIDIS, E. & PATAKAS, D. (2002) Tumor necrosis factor-alpha serum levels, weight loss and tissue oxygenation in chronic obstructive pulmonary disease. *Respir Med,* 96**,** 594-8.

PLATAKI, M., TZORTZAKI, E., RYTILA, P., DEMOSTHENES, M., KOUTSOPOULOS, A. & SIAFAKAS, N. M. (2006) Apoptotic

mechanisms in the pathogenesis of COPD. *Int J Chron Obstruct Pulmon Dis,* 1**,** 161-71.

PLETZ, M. W., IOANAS, M., DE ROUX, A., BURKHARDT, O. & LODE, H. (2004) Reduced spontaneous apoptosis in peripheral blood neutrophils during exacerbation of COPD. *Eur Respir J,* 23**,** 532-7.

PODACK, E. R., YOUNG, J. D. & COHN, Z. A. (1985) Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. *Proc Natl Acad Sci U S A,* 82**,** 8629-33.

PONS, J., SAULEDA, J., FERRER, J. M., BARCELO, B., FUSTER, A., REGUEIRO, V., JULIA, M. R. & AGUSTI, A. G. (2005) Blunted gamma delta T-lymphocyte response in chronic obstructive pulmonary disease. *Eur Respir J,* 25**,** 441-6.

PRAUSE, O., LAAN, M., LOTVALL, J. & LINDEN, A. (2003) Pharmacological modulation of interleukin-17-induced GCP-2-, GRO-alpha- and interleukin-8 release in human bronchial epithelial cells. *Eur J Pharmacol,* 462**,** 193-8.

PREMACK, B. A. & SCHALL, T. J. (1996) Chemokine receptors: gateways to inflammation and infection. *Nat Med,* 2**,** 1174-8.

PRESCOTT, E. & VESTBO, J. (1999) Socioeconomic status and chronic obstructive pulmonary disease. *Thorax,* 54**,** 737-41.

PRIETO, A., REYES, E., BERNSTEIN, E. D., MARTINEZ, B., MONSERRAT, J., IZQUIERDO, J. L., CALLOL, L., DE, L. P., ALVAREZ-SALA, R., ALVAREZ-SALA, J. L., VILLARRUBIA, V. G. & ALVAREZ-MON, M. (2001) Defective natural killer and phagocytic activities in chronic obstructive pulmonary disease are restored by glycophosphopeptical (inmunoferon). *Am J Respir Crit Care Med,* 163**,** 1578-83.

PROFITA, M., CHIAPPARA, G., MIRABELLA, F., DI GIORGI, R., CHIMENTI, L., COSTANZO, G., RICCOBONO, L., BELLIA, V., BOUSQUET, J. & VIGNOLA, A. M. (2003) Effect of cilomilast (Ariflo) on TNF-alpha, IL-8, and GM-CSF release by airway cells of patients with COPD. *Thorax,* 58**,** 573-9.

PROFITA, M., GIORGI, R. D., SALA, A., BONANNO, A., RICCOBONO, L., MIRABELLA, F., GJOMARKAJ, M., BONSIGNORE, G., BOUSQUET, J. & VIGNOLA, A. M. (2005) Muscarinic receptors, leukotriene B4 production and neutrophilic inflammation in COPD patients. *Allergy,* 60**,** 1361-9.

PROUD, D. & CHOW, C. W. (2006) Role of viral infections in asthma and chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol,* 35**,** 513-8.

PROUDFOOT, A. E. (2002) Chemokine receptors: multifaceted therapeutic targets. *Nat Rev Immunol,* 2**,** 106-15.

PRYOR, W. A. & STONE, K. (1993) Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. *Ann N Y Acad Sci,* 686**,** 12-27; discussion 27-8.

PUCHELLE, E., ZAHM, J. M., TOURNIER, J. M. & CORAUX, C. (2006) Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease. *Proc Am Thorac Soc,* 3**,** 726-33.

QIU, Y., ZHU, J., BANDI, V., ATMAR, R. L., HATTOTUWA, K., GUNTUPALLI, K. K. & JEFFERY, P. K. (2003) Biopsy neutrophilia,

neutrophil chemokine and receptor gene expression in severe exacerbations of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 168**,** 968-75.

QU, N., XU, M., MIZOGUCHI, I., FURUSAWA, J., KANEKO, K., WATANABE, K., MIZUGUCHI, J., ITOH, M., KAWAKAMI, Y. & YOSHIMOTO, T. (2013) Pivotal Roles of T-Helper 17-Related Cytokines, IL-17, IL-22, and IL-23, in Inflammatory Diseases. *Clin Dev Immunol,* 2013**,** 968549.

RABE, K. F., HURD, S., ANZUETO, A., BARNES, P. J., BUIST, S. A., CALVERLEY, P., FUKUCHI, Y., JENKINS, C., RODRIGUEZ-ROISIN, R., VAN WEEL, C. & ZIELINSKI, J. (2007) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med,* 176**,** 532-55.

RABINOVICH, R. A., FIGUERAS, M., ARDITE, E., CARBO, N., TROOSTERS, T., FILELLA, X., BARBERA, J. A., FERNANDEZ-CHECA, J. C., ARGILES, J. M. & ROCA, J. (2003) Increased tumour necrosis factor-alpha plasma levels during moderate-intensity exercise in COPD patients. *Eur Respir J,* 21**,** 789-94.

RADAEV, S., MOTYKA, S., FRIDMAN, W. H., SAUTES-FRIDMAN, C. & SUN, P. D. (2001) The structure of a human type III Fcgamma receptor in complex with Fc. *J Biol Chem,* 276**,** 16469-77.

RAHMAN, I., MORRISON, D., DONALDSON, K. & MACNEE, W. (1996) Systemic oxidative stress in asthma, COPD, and smokers. *Am J Respir Crit Care Med,* 154**,** 1055-60.

RAHMAN, I., VAN SCHADEWIJK, A. A., CROWTHER, A. J., HIEMSTRA, P. S., STOLK, J., MACNEE, W. & DE BOER, W. I. (2002) 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 166**,** 490-5.

RAJAGOPALAN, S. & LONG, E. O. (1999) A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells. *J Exp Med,* 189**,** 1093-100.

RAMOS, C., CISNEROS, J., GONZALEZ-AVILA, G., BECERRIL, C., RUIZ, V. & MONTANO, M. (2009) Increase of matrix metalloproteinases in woodsmoke-induced lung emphysema in guinea pigs. *Inhal Toxicol,* 21**,** 119-32.

RANES, J. & STOLLER, J. K. (2005) A review of alpha-1 antitrypsin deficiency. *Semin Respir Crit Care Med,* 26**,** 154-66.

RAULET, D. H. (2003) Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol,* 3**,** 781-90.

RAULET, D. H., VANCE, R. E. & MCMAHON, C. W. (2001) Regulation of the natural killer cell receptor repertoire. *Annu Rev Immunol,* 19**,** 291-330.

READER, J. R., HYDE, D. M., SCHELEGLE, E. S., ALDRICH, M. C., STODDARD, A. M., MCLANE, M. P., LEVITT, R. C. & TEPPER, J. S. (2003) Interleukin-9 induces mucous cell metaplasia independent of inflammation. *Am J Respir Cell Mol Biol,* 28**,** 664-72.

REGNER, M. & MULLBACHER, A. (2004) Granzymes in cytolytic lymphocytes--to kill a killer? *Immunol Cell Biol,* 82**,** 161-9.

REIBMAN, J., HSU, Y., CHEN, L. C., BLECK, B. & GORDON, T. (2003) Airway epithelial cells release MIP-3alpha/CCL20 in response to cytokines and ambient particulate matter. *Am J Respir Cell Mol Biol,* 28**,** 648-54.

REPINE, J. E., BAST, A. & LANKHORST, I. (1997) Oxidative stress in chronic obstructive pulmonary disease. Oxidative Stress Study Group. *Am J Respir Crit Care Med,* 156**,** 341-57.

REYMOND, N., IMBERT, A. M., DEVILARD, E., FABRE, S., CHABANNON, C., XERRI, L., FARNARIER, C., CANTONI, C., BOTTINO, C., MORETTA, A., DUBREUIL, P. & LOPEZ, M. (2004) DNAM-1 and PVR regulate monocyte migration through endothelial junctions. *J Exp Med,* 199**,** 1331-41.

REYNOLDS, P. R., COSIO, M. G. & HOIDAL, J. R. (2006) Cigarette smoke-induced Egr-1 upregulates proinflammatory cytokines in pulmonary epithelial cells. *Am J Respir Cell Mol Biol,* 35**,** 314-9.

RICHMOND, I., PRITCHARD, G. E., ASHCROFT, T., CORRIS, P. A. & WALTERS, E. H. (1993) Distribution of gamma delta T-cells in the bronchial tree of smokers and non-smokers. *J Clin Pathol,* 46**,** 926-30.

ROGERS, W. O., WEAVER, C. T., KRAUS, L. A., LI, J., LI, L. & BUCY, R. P. (1997) Visualization of antigen-specific T cell activation and cytokine expression in vivo. *J Immunol,* 158**,** 649-57.

ROHDE, G., WIETHEGE, A., BORG, I., KAUTH, M., BAUER, T. T., GILLISSEN, A., BUFE, A. & SCHULTZE-WERNINGHAUS, G. (2003) Respiratory viruses in exacerbations of chronic obstructive pulmonary disease requiring hospitalisation: a case-control study. *Thorax,* 58**,** 37-42.

ROOS-ENGSTRAND, E., EKSTRAND-HAMMARSTROM, B., POURAZAR, J., BEHNDIG, A. F., BUCHT, A. & BLOMBERG, A. (2009) Influence of smoking cessation on airway T lymphocyte subsets in COPD. *Copd,* 6**,** 112-20.

ROOS-ENGSTRAND, E., POURAZAR, J., BEHNDIG, A. F., BLOMBERG, A. & BUCHT, A. (2010) Cytotoxic T cells expressing the co-stimulatory receptor NKG2 D are increased in cigarette smoking and COPD. *Respir Res,* 11**,** 128.

ROSE, C. E., JR., SUNG, S. S. & FU, S. M. (2003) Significant involvement of CCL2 (MCP-1) in inflammatory disorders of the lung. *Microcirculation,* 10**,** 273-88.

ROSEN, D. B., ARAKI, M., HAMERMAN, J. A., CHEN, T., YAMAMURA, T. & LANIER, L. L. (2004) A Structural basis for the association of DAP12 with mouse, but not human, NKG2D. *J Immunol,* 173**,** 2470-8.

ROSENBERG, S. A., LOTZE, M. T., MUUL, L. M., CHANG, A. E., AVIS, F. P., LEITMAN, S., LINEHAN, W. M., ROBERTSON, C. N., LEE, R. E., RUBIN, J. T. & ET AL. (1987) A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med,* 316**,** 889-97.

ROVINA, N., DIMA, E., GERASSIMOU, C., KOLLINTZA, A., GRATZIOU, C. & ROUSSOS, C. (2009) Interleukin-18 in induced sputum: association with lung function in chronic obstructive pulmonary disease. *Respir Med,* 103**,** 1056-62.

RUSSELL, J. H. & LEY, T. J. (2002) Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol,* 20**,** 323-70.

RUSSELL, R. E., CULPITT, S. V., DEMATOS, C., DONNELLY, L., SMITH, M., WIGGINS, J. & BARNES, P. J. (2002) Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol,* 26**,** 602-9.

RUSSO, C. & POLOSA, R. (2005) TNF-alpha as a promising therapeutic target in chronic asthma: a lesson from rheumatoid arthritis. *Clin Sci (Lond),* 109**,** 135-42.

RUSZNAK, C., DEVALIA, J. L., SAPSFORD, R. J. & DAVIES, R. J. (1996) Ozone-induced mediator release from human bronchial epithelial cells in vitro and the influence of nedocromil sodium. *Eur Respir J,* 9**,** 2298-305.

RUSZNAK, C., MILLS, P. R., DEVALIA, J. L., SAPSFORD, R. J., DAVIES, R. J. & LOZEWICZ, S. (2000) Effect of cigarette smoke on the permeability and IL-1beta and sICAM-1 release from cultured human bronchial epithelial cells of never-smokers, smokers, and patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol,* 23**,** 530-6.

SAETTA, M., BARALDO, S., CORBINO, L., TURATO, G., BRACCIONI, F., REA, F., CAVALLESCO, G., TROPEANO, G., MAPP, C. E., MAESTRELLI, P., CIACCIA, A. & FABBRI, L. M. (1999) CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 160**,** 711-7.

SAETTA, M., BARALDO, S., TURATO, G., BEGHE, B., CASONI, G. L., BELLETTATO, C. M., REA, F., ZUIN, R., FABBRI, L. M. & PAPI, A. (2003) Increased proportion of CD8+ T-lymphocytes in the paratracheal lymph nodes of smokers with mild COPD. *Sarcoidosis Vasc Diffuse Lung Dis,* 20**,** 28-32.

SAETTA, M., DI STEFANO, A., MAESTRELLI, P., FERRARESSO, A., DRIGO, R., POTENA, A., CIACCIA, A. & FABBRI, L. M. (1993) Activated T-lymphocytes and macrophages in bronchial mucosa of subjects with chronic bronchitis. *Am Rev Respir Dis,* 147**,** 301-6.

SAETTA, M., DI STEFANO, A., MAESTRELLI, P., TURATO, G., MAPP, C. E., PIENO, M., ZANGUOCHI, G., DEL PRETE, G. & FABBRI, L. M. (1996) Airway eosinophilia and expression of interleukin-5 protein in asthma and in exacerbations of chronic bronchitis. *Clin Exp Allergy,* 26**,** 766-74.

SAETTA, M., DI STEFANO, A., MAESTRELLI, P., TURATO, G., RUGGIERI, M. P., ROGGERI, A., CALCAGNI, P., MAPP, C. E., CIACCIA, A. & FABBRI, L. M. (1994) Airway eosinophilia in chronic bronchitis during exacerbations. *Am J Respir Crit Care Med,* 150**,** 1646-52.

SAETTA, M., DI STEFANO, A., TURATO, G., FACCHINI, F. M., CORBINO, L., MAPP, C. E., MAESTRELLI, P., CIACCIA, A. & FABBRI, L. M. (1998) CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 157**,** 822-6.

SAETTA, M., MARIANI, M., PANINA-BORDIGNON, P., TURATO, G., BUONSANTI, C., BARALDO, S., BELLETTATO, C. M., PAPI, A., CORBETTA, L., ZUIN, R., SINIGAGLIA, F. & FABBRI, L. M. (2002) Increased expression of the chemokine receptor CXCR3 and its ligand CXCL10 in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 165**,** 1404-9.

SAETTA, M., TURATO, G., FACCHINI, F. M., CORBINO, L., LUCCHINI, R. E., CASONI, G., MAESTRELLI, P., MAPP, C. E., CIACCIA, A. & FABBRI, L. M. (1997) Inflammatory cells in the bronchial glands of smokers with chronic bronchitis. *Am J Respir Crit Care Med,* 156**,** 1633-9.

SAHIRATMADJA, E., ALISJAHBANA, B., BUCCHERI, S., DI LIBERTO, D., DE BOER, T., ADNAN, I., VAN CREVEL, R., KLEIN, M. R., VAN MEIJGAARDEN, K. E., NELWAN, R. H., VAN DE VOSSE, E., DIELI, F. & OTTENHOFF, T. H. (2007) Plasma granulysin levels and cellular interferon-gamma production correlate with curative host responses in tuberculosis, while plasma interferon-gamma levels correlate with tuberculosis disease activity in adults. *Tuberculosis (Edinb),* 87**,** 312-21.

SALVI, S. S. & BARNES, P. J. (2009) Chronic obstructive pulmonary disease in non-smokers. *Lancet,* 374**,** 733-43.

SANDHAUS, R. A. (2009) Augmentation therapy in alpha-1 antitrypsin deficiency. *Copd,* 6**,** 147-8.

SANTOS, S., PEINADO, V. I., RAMIREZ, J., MORALES-BLANHIR, J., BASTOS, R., ROCA, J., RODRIGUEZ-ROISIN, R. & BARBERA, J. A. (2003) Enhanced expression of vascular endothelial growth factor in pulmonary arteries of smokers and patients with moderate chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 167**,** 1250-6.

SAPEY, E., AHMAD, A., BAYLEY, D., NEWBOLD, P., SNELL, N., RUGMAN, P. & STOCKLEY, R. A. (2009) Imbalances between interleukin-1 and tumor necrosis factor agonists and antagonists in stable COPD. *J Clin Immunol,* 29**,** 508-16.

SAWYER, R. T. (1986) The significance of local resident pulmonary alveolar macrophage proliferation to population renewal. *J Leukoc Biol,* 39**,** 77-87.

SCANLON, P. D., CONNETT, J. E., WALLER, L. A., ALTOSE, M. D., BAILEY, W. C., BUIST, A. S. & TASHKIN, D. P. (2000) Smoking cessation and lung function in mild-to-moderate chronic obstructive pulmonary disease. The Lung Health Study. *Am J Respir Crit Care Med,* 161**,** 381-90.

SCAPINI, P., CARLETTO, A., NARDELLI, B., CALZETTI, F., ROSCHKE, V., MERIGO, F., TAMASSIA, N., PIEROPAN, S., BIASI, D., SBARBATI, A., SOZZANI, S., BAMBARA, L. & CASSATELLA, M. A. (2005) Proinflammatory mediators elicit secretion of the intracellular B-lymphocyte stimulator pool (BLyS) that is stored in activated neutrophils: implications for inflammatory diseases. *Blood,* 105**,** 830-7.

SCHAERLI, P., WILLIMANN, K., LANG, A. B., LIPP, M., LOETSCHER, P. & MOSER, B. (2000) CXC chemokine receptor 5 expression defines

follicular homing T cells with B cell helper function. *J Exp Med,* 192**,** 1553-62.

SCHMIDT-IOANAS, M., PLETZ, M. W., DE ROUX, A. & LODE, H. (2006) Apoptosis of peripheral blood neutrophils in COPD exacerbation does not correlate with serum cytokines. *Respir Med,* 100**,** 639-47.

SCHMIDT-WOLF, I. G., LEFTEROVA, P., MEHTA, B. A., FERNANDEZ, L. P., HUHN, D., BLUME, K. G., WEISSMAN, I. L. & NEGRIN, R. S. (1993) Phenotypic characterization and identification of effector cells involved in tumor cell recognition of cytokine-induced killer cells. *Exp Hematol,* 21**,** 1673-9.

SCHOLS, A. M., BUURMAN, W. A., STAAL VAN DEN BREKEL, A. J., DENTENER, M. A. & WOUTERS, E. F. (1996) Evidence for a relation between metabolic derangements and increased levels of inflammatory mediators in a subgroup of patients with chronic obstructive pulmonary disease. *Thorax,* 51**,** 819-24.

SCHULZ, C., KRATZEL, K., WOLF, K., SCHROLL, S., KOHLER, M. & PFEIFER, M. (2004) Activation of bronchial epithelial cells in smokers without airway obstruction and patients with COPD. *Chest,* 125**,** 1706-13.

SCHULZ, C., WOLF, K., HARTH, M., KRATZEL, K., KUNZ-SCHUGHART, L. & PFEIFER, M. (2003) Expression and release of interleukin-8 by human bronchial epithelial cells from patients with chronic obstructive pulmonary disease, smokers, and never-smokers. *Respiration,* 70**,** 254-61.

SCIURBA, F. C. (2004) Physiologic similarities and differences between COPD and asthma. *Chest,* 126**,** 117S-124S; discussion 159S-161S.

SCOTT, J. L., DUNN, S. M., JIN, B., HILLAM, A. J., WALTON, S., BERNDT, M. C., MURRAY, A. W., KRISSANSEN, G. W. & BURNS, G. F. (1989) Characterization of a novel membrane glycoprotein involved in platelet activation. *J Biol Chem,* 264**,** 13475-82.

SEEMUNGAL, T., HARPER-OWEN, R., BHOWMIK, A., MORIC, I., SANDERSON, G., MESSAGE, S., MACCALLUM, P., MEADE, T. W., JEFFRIES, D. J., JOHNSTON, S. L. & WEDZICHA, J. A. (2001) Respiratory viruses, symptoms, and inflammatory markers in acute exacerbations and stable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 164**,** 1618-23.

SEGURA-VALDEZ, L., PARDO, A., GAXIOLA, M., UHAL, B. D., BECERRIL, C. & SELMAN, M. (2000) Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD. *Chest,* 117**,** 684-94.

SEKIZAWA, K., CAUGHEY, G. H., LAZARUS, S. C., GOLD, W. M. & NADEL, J. A. (1989) Mast cell tryptase causes airway smooth muscle hyperresponsiveness in dogs. *J Clin Invest,* 83**,** 175-9.

SELMAN, M., CISNEROS-LIRA, J., GAXIOLA, M., RAMIREZ, R., KUDLACZ, E. M., MITCHELL, P. G. & PARDO, A. (2003) Matrix metalloproteinases inhibition attenuates tobacco smoke-induced emphysema in Guinea pigs. *Chest,* 123**,** 1633-41.

SERTL, K., TAKEMURA, T., TSCHACHLER, E., FERRANS, V. J., KALINER, M. A. & SHEVACH, E. M. (1986) Dendritic cells with

antigen-presenting capability reside in airway epithelium, lung parenchyma, and visceral pleura. *J Exp Med,* 163**,** 436-51.

SETHI, S., SETHI, R., ESCHBERGER, K., LOBBINS, P., CAI, X., GRANT, B. J. & MURPHY, T. F. (2007) Airway bacterial concentrations and exacerbations of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 176**,** 356-61.

SHAMSUDDIN, M., CHEN, E., ANDERSON, J. & SMITH, L. J. (1997) Regulation of leukotriene and platelet-activating factor synthesis in human alveolar macrophages. *J Lab Clin Med,* 130**,** 615-26.

SHAPIRO, S. D. (1999) The macrophage in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 160**,** S29-32.

SHARIF, S., ARREAZA, G. A., ZUCKER, P., MI, Q. S. & DELOVITCH, T. L. (2002) Regulation of autoimmune disease by natural killer T cells. *J Mol Med (Berl),* 80**,** 290-300.

SHEN, L. S., WANG, J., SHEN, D. F., YUAN, X. L., DONG, P., LI, M. X., XUE, J., ZHANG, F. M., GE, H. L. & XU, D. (2009) CD4(+)CD25(+)CD127(low/-) regulatory T cells express Foxp3 and suppress effector T cell proliferation and contribute to gastric cancers progression. *Clin Immunol,* 131**,** 109-18.

SHI, L., MAI, S., ISRAELS, S., BROWNE, K., TRAPANI, J. A. & GREENBERG, A. H. (1997) Granzyme B (GraB) autonomously crosses the cell membrane and perforin initiates apoptosis and GraB nuclear localization. *J Exp Med,* 185**,** 855-66.

SHIBUYA, A., CAMPBELL, D., HANNUM, C., YSSEL, H., FRANZ-BACON, K., MCCLANAHAN, T., KITAMURA, T., NICHOLL, J., SUTHERLAND, G. R., LANIER, L. L. & PHILLIPS, J. H. (1996) DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. *Immunity,* 4**,** 573-81.

SHIBUYA, A., NAGAYOSHI, K., NAKAMURA, K. & NAKAUCHI, H. (1995) Lymphokine requirement for the generation of natural killer cells from CD34+ hematopoietic progenitor cells. *Blood,* 85**,** 3538-46.

SHIM, J. J., DABBAGH, K., UEKI, I. F., DAO-PICK, T., BURGEL, P. R., TAKEYAMA, K., TAM, D. C. & NADEL, J. A. (2001) IL-13 induces mucin production by stimulating epidermal growth factor receptors and by activating neutrophils. *Am J Physiol Lung Cell Mol Physiol,* 280**,** L134-40.

SHIMBARA, A., CHRISTODOULOPOULOS, P., SOUSSI-GOUNNI, A., OLIVENSTEIN, R., NAKAMURA, Y., LEVITT, R. C., NICOLAIDES, N. C., HOLROYD, K. J., TSICOPOULOS, A., LAFITTE, J. J., WALLAERT, B. & HAMID, Q. A. (2000) IL-9 and its receptor in allergic and nonallergic lung disease: increased expression in asthma. *J Allergy Clin Immunol,* 105**,** 108-15.

SHRESTA, S., GRAUBERT, T. A., THOMAS, D. A., RAPTIS, S. Z. & LEY, T. J. (1999) Granzyme A initiates an alternative pathway for granule-mediated apoptosis. *Immunity,* 10**,** 595-605.

SHUTO, T., XU, H., WANG, B., HAN, J., KAI, H., GU, X. X., MURPHY, T. F., LIM, D. J. & LI, J. D. (2001) Activation of NF-kappa B by nontypeable Hemophilus influenzae is mediated by toll-like receptor 2-TAK1-dependent NIK-IKK alpha /beta-I kappa B alpha and MKK3/6-

p38 MAP kinase signaling pathways in epithelial cells. *Proc Natl Acad Sci U S A,* 98**,** 8774-9.

SILVERMAN, E. K., WEISS, S. T., DRAZEN, J. M., CHAPMAN, H. A., CAREY, V., CAMPBELL, E. J., DENISH, P., SILVERMAN, R. A., CELEDON, J. C., REILLY, J. J., GINNS, L. C. & SPEIZER, F. E. (2000) Gender-related differences in severe, early-onset chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 162**,** 2152-8.

SINGH, D., EDWARDS, L., TAL-SINGER, R. & RENNARD, S. (2010) Sputum neutrophils as a biomarker in COPD: findings from the ECLIPSE study. *Respir Res,* 11**,** 77.

SKILLRUD, D. M., OFFORD, K. P. & MILLER, R. D. (1986) Higher risk of lung cancer in chronic obstructive pulmonary disease. A prospective, matched, controlled study. *Ann Intern Med,* 105**,** 503-7.

SKOLD, C. M., LUNDAHL, J., HALLDEN, G., HALLGREN, M. & EKLUND, A. (1996) Chronic smoke exposure alters the phenotype pattern and the metabolic response in human alveolar macrophages. *Clin Exp Immunol,* 106**,** 108-13.

SKOLD, M. & BEHAR, S. M. (2003) Role of CD1d-restricted NKT cells in microbial immunity. *Infect Immun,* 71**,** 5447-55.

SLEVOGT, H., SCHMECK, B., JONATAT, C., ZAHLTEN, J., BEERMANN, W., VAN LAAK, V., OPITZ, B., DIETEL, S., N'GUESSAN, P. D., HIPPENSTIEL, S., SUTTORP, N. & SEYBOLD, J. (2006) Moraxella catarrhalis induces inflammatory response of bronchial epithelial cells via MAPK and NF-kappaB activation and histone deacetylase activity reduction. *Am J Physiol Lung Cell Mol Physiol,* 290**,** L818-26.

SMITH, C. A., FARRAH, T. & GOODWIN, R. G. (1994) The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell,* 76**,** 959-62.

SMITH, K. A. (1988a) The bimolecular structure of the interleukin 2 receptor. *Immunol Today,* 9**,** 36-7.

SMITH, K. A. (1988b) The interleukin 2 receptor. *Adv Immunol,* 42**,** 165-79.

SMYTH, L. J., STARKEY, C., VESTBO, J. & SINGH, D. (2007) CD4-regulatory cells in COPD patients. *Chest,* 132**,** 156-63.

SMYTH, M. J., THIA, K. Y., STREET, S. E., CRETNEY, E., TRAPANI, J. A., TANIGUCHI, M., KAWANO, T., PELIKAN, S. B., CROWE, N. Y. & GODFREY, D. I. (2000) Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med,* 191**,** 661-8.

SMYTH, M. J., WALLACE, M. E., NUTT, S. L., YAGITA, H., GODFREY, D. I. & HAYAKAWA, Y. (2005) Sequential activation of NKT cells and NK cells provides effective innate immunotherapy of cancer. *J Exp Med,* 201**,** 1973-85.

SNYDER, M. R., LUCAS, M., VIVIER, E., WEYAND, C. M. & GORONZY, J. J. (2003) Selective activation of the c-Jun NH2-terminal protein kinase signaling pathway by stimulatory KIR in the absence of KARAP/DAP12 in CD4+ T cells. *J Exp Med,* 197**,** 437-49.

SOLER, N., EWIG, S., TORRES, A., FILELLA, X., GONZALEZ, J. & ZAUBET, A. (1999) Airway inflammation and bronchial microbial patterns in patients with stable chronic obstructive pulmonary disease. *Eur Respir J,* 14**,** 1015-22.

SOLER, P., MOREAU, A., BASSET, F. & HANCE, A. J. (1989) Cigarette smoking-induced changes in the number and differentiated state of pulmonary dendritic cells/Langerhans cells. *Am Rev Respir Dis,* 139**,** 1112-7.

SOLTANI, A., EWE, Y. P., LIM, Z. S., SOHAL, S. S., REID, D., WESTON, S., WOOD-BAKER, R. & WALTERS, E. H. (2012) Mast cells in COPD airways: relationship to bronchodilator responsiveness and angiogenesis. *Eur Respir J,* 39**,** 1361-7.

SOMMERHOFF, C. P., NADEL, J. A., BASBAUM, C. B. & CAUGHEY, G. H. (1990) Neutrophil elastase and cathepsin G stimulate secretion from cultured bovine airway gland serous cells. *J Clin Invest,* 85**,** 682-9.

SOPORI, M. (2002) Effects of cigarette smoke on the immune system. *Nat Rev Immunol,* 2**,** 372-7.

SORIANO, J. B., MAIER, W. C., EGGER, P., VISICK, G., THAKRAR, B., SYKES, J. & PRIDE, N. B. (2000) Recent trends in physician diagnosed COPD in women and men in the UK. *Thorax,* 55**,** 789-94.

SPEISER, D. E., COLONNA, M., AYYOUB, M., CELLA, M., PITTET, M. J., BATARD, P., VALMORI, D., GUILLAUME, P., LIENARD, D., CEROTTINI, J. C. & ROMERO, P. (2001) The activatory receptor 2B4 is expressed in vivo by human CD8+ effector alpha beta T cells. *J Immunol,* 167**,** 6165-70.

SPICUZZA, L., GIEMBYCZ, M. A., BARNES, P. J. & BELVISI, M. G. (1998) Prostaglandin E2 suppression of acetylcholine release from parasympathetic nerves innervating guinea-pig trachea by interacting with prostanoid receptors of the EP3-subtype. *Br J Pharmacol,* 123**,** 1246-52.

SPRUIT, M. A., GOSSELINK, R., TROOSTERS, T., KASRAN, A., GAYAN-RAMIREZ, G., BOGAERTS, P., BOUILLON, R. & DECRAMER, M. (2003) Muscle force during an acute exacerbation in hospitalised patients with COPD and its relationship with CXCL8 and IGF-I. *Thorax,* 58**,** 752-6.

STANESCU, D., SANNA, A., VERITER, C., KOSTIANEV, S., CALCAGNI, P. G., FABBRI, L. M. & MAESTRELLI, P. (1996) Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. *Thorax,* 51**,** 267-71.

STANNARD, W. & O'CALLAGHAN, C. (2006) Ciliary function and the role of cilia in clearance. *J Aerosol Med,* 19**,** 110-5.

STEDMAN, R. L. (1968) The chemical composition of tobacco and tobacco smoke. *Chem Rev,* 68**,** 153-207.

STENSTROM, M., SKOLD, M., ERICSSON, A., BEAUDOIN, L., SIDOBRE, S., KRONENBERG, M., LEHUEN, A. & CARDELL, S. (2004) Surface receptors identify mouse NK1.1+ T cell subsets distinguished by function and T cell receptor type. *Eur J Immunol,* 34**,** 56-65.

STINCHCOMBE, J. C., BOSSI, G., BOOTH, S. & GRIFFITHS, G. M. (2001) The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity,* 15**,** 751-61.

STOLL, P., HEINZ, A. S., BRATKE, K., BIER, A., GARBE, K., KUEPPER, M., VIRCHOW, J. C. & LOMMATZSCH, M. (2014) Impact of

smoking on dendritic cell phenotypes in the airway lumen of patients with COPD. *Respir Res,* 15**,** 48.

STONE, R., BARNES, P. J. & FULLER, R. W. (1992) Contrasting effects of prostaglandins E2 and F2 alpha on sensitivity of the human cough reflex. *J Appl Physiol,* 73**,** 649-53.

STRANGE, C. (2013) Airway disease in alpha-1 antitrypsin deficiency. *Copd,* 10 Suppl 1**,** 68-73.

STRINGER, K. A., TOBIAS, M., O'NEILL, H. C. & FRANKLIN, C. C. (2007) Cigarette smoke extract-induced suppression of caspase-3-like activity impairs human neutrophil phagocytosis. *Am J Physiol Lung Cell Mol Physiol,* 292**,** L1572-9.

SUBRAMANIAM, S., WHITSETT, J. A., HULL, W. & GAIROLA, C. G. (1996) Alteration of pulmonary surfactant proteins in rats chronically exposed to cigarette smoke. *Toxicol Appl Pharmacol,* 140**,** 274-80.

SUDA, T., OKAZAKI, T., NAITO, Y., YOKOTA, T., ARAI, N., OZAKI, S., NAKAO, K. & NAGATA, S. (1995) Expression of the Fas ligand in cells of T cell lineage. *J Immunol,* 154**,** 3806-13.

SUDA, T., TAKAHASHI, T., GOLSTEIN, P. & NAGATA, S. (1993) Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell,* 75**,** 1169-78.

SUNYER, J. (2001) Urban air pollution and chronic obstructive pulmonary disease: a review. *Eur Respir J,* 17**,** 1024-33.

SUZUKI, N., SUZUKI, T. & ENGLEMAN, E. G. (1991) Evidence for the involvement of CD56 molecules in alloantigen-specific recognition by human natural killer cells. *J Exp Med,* 173**,** 1451-61.

SUZUKI, Y., HAMAMOTO, Y., OGASAWARA, Y., ISHIKAWA, K., YOSHIKAWA, Y., SASAZUKI, T. & MUTO, M. (2004) Genetic polymorphisms of killer cell immunoglobulin-like receptors are associated with susceptibility to psoriasis vulgaris. *J Invest Dermatol,* 122**,** 1133-6.

TAHARA-HANAOKA, S., SHIBUYA, K., ONODA, Y., ZHANG, H., YAMAZAKI, S., MIYAMOTO, A., HONDA, S., LANIER, L. L. & SHIBUYA, A. (2004) Functional characterization of DNAM-1 (CD226) interaction with its ligands PVR (CD155) and nectin-2 (PRR-2/CD112). *Int Immunol,* 16**,** 533-8.

TAKABATAKE, N., NAKAMURA, H., INOUE, S., TERASHITA, K., YUKI, H., KATO, S., YASUMURA, S. & TOMOIKE, H. (2000) Circulating levels of soluble Fas ligand and soluble Fas in patients with chronic obstructive pulmonary disease. *Respir Med,* 94**,** 1215-20.

TAKAHASHI, H. K., IWAGAKI, H., HAMANO, R., KANKE, T., LIU, K., SADAMORI, H., YAGI, T., YOSHINO, T., TANAKA, N. & NISHIBORI, M. (2007) The immunosuppressive effects of nicotine during human mixed lymphocyte reaction. *Eur J Pharmacol,* 559**,** 69-74.

TAKAHASHI, H. K., IWAGAKI, H., HAMANO, R., YOSHINO, T., TANAKA, N. & NISHIBORI, M. (2006) Effect of nicotine on IL-18-initiated immune response in human monocytes. *J Leukoc Biol,* 80**,** 1388-94.

TAKAHASHI, T., CHIBA, S., NIEDA, M., AZUMA, T., ISHIHARA, S., SHIBATA, Y., JUJI, T. & HIRAI, H. (2002) Cutting edge: analysis of

human V alpha 24+CD8+ NK T cells activated by alpha-galactosylceramide-pulsed monocyte-derived dendritic cells. *J Immunol,* 168**,** 3140-4.

TAKAHASHI, T., NIEDA, M., KOEZUKA, Y., NICOL, A., PORCELLI, S. A., ISHIKAWA, Y., TADOKORO, K., HIRAI, H. & JUJI, T. (2000) Analysis of human V alpha 24+ CD4+ NKT cells activated by alpha-glycosylceramide-pulsed monocyte-derived dendritic cells. *J Immunol,* 164**,** 4458-64.

TAKEUCHI, M., NAGAI, S. & IZUMI, T. (1988) Effect of smoking on natural killer cell activity in the lung. *Chest,* 94**,** 688-93.

TAKIZAWA, H., TANAKA, M., TAKAMI, K., OHTOSHI, T., ITO, K., SATOH, M., OKADA, Y., YAMASAWA, F., NAKAHARA, K. & UMEDA, A. (2001) Increased expression of transforming growth factor-beta1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD). *Am J Respir Crit Care Med,* 163**,** 1476-83.

TALANIAN, R. V., YANG, X., TURBOV, J., SETH, P., GHAYUR, T., CASIANO, C. A., ORTH, K. & FROELICH, C. J. (1997) Granule-mediated killing: pathways for granzyme B-initiated apoptosis. *J Exp Med,* 186**,** 1323-31.

TANG, G., MINEMOTO, Y., DIBLING, B., PURCELL, N. H., LI, Z., KARIN, M. & LIN, A. (2001) Inhibition of JNK activation through NF-kappaB target genes. *Nature,* 414**,** 313-7.

TANG, Q. & BLUESTONE, J. A. (2008) The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol,* 9**,** 239-44.

TANGYE, S. G., PHILLIPS, J. H. & LANIER, L. L. (2000) The CD2-subset of the Ig superfamily of cell surface molecules: receptor-ligand pairs expressed by NK cells and other immune cells. *Semin Immunol,* 12**,** 149-57.

TANIGUCHI, T. & MINAMI, Y. (1993) The IL-2/IL-2 receptor system: a current overview. *Cell,* 73**,** 5-8.

TANINO, M., BETSUYAKU, T., TAKEYABU, K., TANINO, Y., YAMAGUCHI, E., MIYAMOTO, K. & NISHIMURA, M. (2002) Increased levels of interleukin-8 in BAL fluid from smokers susceptible to pulmonary emphysema. *Thorax,* 57**,** 405-11.

TARLING, J. D., LIN, H. S. & HSU, S. (1987) Self-renewal of pulmonary alveolar macrophages: evidence from radiation chimera studies. *J Leukoc Biol,* 42**,** 443-6.

TASHKIN, D. P. & MURRAY, R. P. (2009) Smoking cessation in chronic obstructive pulmonary disease. *Respir Med,* 103**,** 963-74.

TAYLOR, A. E., FINNEY-HAYWARD, T. K., QUINT, J. K., THOMAS, C. M., TUDHOPE, S. J., WEDZICHA, J. A., BARNES, P. J. & DONNELLY, L. E. (2010) Defective macrophage phagocytosis of bacteria in COPD. *Eur Respir J,* 35**,** 1039-47.

TERABE, M., MATSUI, S., NOBEN-TRAUTH, N., CHEN, H., WATSON, C., DONALDSON, D. D., CARBONE, D. P., PAUL, W. E. & BERZOFSKY, J. A. (2000) NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol,* 1**,** 515-20.

THOMAS, P. S., SCHRECK, R. E. & LAZARUS, S. C. (1992) Tobacco smoke releases performed mediators from canine mast cells and modulates prostaglandin production. *Am J Physiol,* 263**,** L67-72.

THOMAS, R. M. (2008) The biology of Interleukin-2. *Annu Rev Immunol,* 26**,** 453-79.

THOMPSON, C. B., LINDSTEN, T., LEDBETTER, J. A., KUNKEL, S. L., YOUNG, H. A., EMERSON, S. G., LEIDEN, J. M. & JUNE, C. H. (1989) CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc Natl Acad Sci U S A,* 86**,** 1333-7.

TOMITA, K., CARAMORI, G., LIM, S., ITO, K., HANAZAWA, T., OATES, T., CHISELITA, I., JAZRAWI, E., CHUNG, K. F., BARNES, P. J. & ADCOCK, I. M. (2002) Increased p21(CIP1/WAF1) and B cell lymphoma leukemia-x(L) expression and reduced apoptosis in alveolar macrophages from smokers. *Am J Respir Crit Care Med,* 166**,** 724-31.

TRAVES, S. L., CULPITT, S. V., RUSSELL, R. E., BARNES, P. J. & DONNELLY, L. E. (2002) Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. *Thorax,* 57**,** 590-5.

TRAVES, S. L. & DONNELLY, L. E. (2008) Th17 cells in airway diseases. *Curr Mol Med,* 8**,** 416-26.

TRAVES, S. L., SMITH, S. J., BARNES, P. J. & DONNELLY, L. E. (2004) Specific CXC but not CC chemokines cause elevated monocyte migration in COPD: a role for CXCR2. *J Leukoc Biol,* 76**,** 441-50.

TRINCHIERI, G. (2003) The choices of a natural killer. *Nat Immunol,* 4**,** 509-10.

TRINCHIERI, G. & SCOTT, P. (1995) Interleukin-12: a proinflammatory cytokine with immunoregulatory functions. *Res Immunol,* 146**,** 423-31.

TSCHOPP, J., MASSON, D. & STANLEY, K. K. (1986) Structural/functional similarity between proteins involved in complement- and cytotoxic T-lymphocyte-mediated cytolysis. *Nature,* 322**,** 831-4.

TSUKAGOSHI, H., HADDAD, E. B., BARNES, P. J. & CHUNG, K. F. (1995) Bradykinin receptor subtypes in rat lung: effect of interleukin-1 beta. *J Pharmacol Exp Ther,* 273**,** 1257-63.

TUPIN, E., NICOLETTI, A., ELHAGE, R., RUDLING, M., LJUNGGREN, H. G., HANSSON, G. K. & BERNE, G. P. (2004) CD1d-dependent activation of NKT cells aggravates atherosclerosis. *J Exp Med,* 199**,** 417-22.

TURATO, G., ZUIN, R., MINIATI, M., BARALDO, S., REA, F., BEGHE, B., MONTI, S., FORMICHI, B., BOSCHETTO, P., HARARI, S., PAPI, A., MAESTRELLI, P., FABBRI, L. M. & SAETTA, M. (2002) Airway inflammation in severe chronic obstructive pulmonary disease: relationship with lung function and radiologic emphysema. *Am J Respir Crit Care Med,* 166**,** 105-10.

TURATO, G., ZUIN, R. & SAETTA, M. (2001) Pathogenesis and pathology of COPD. *Respiration,* 68**,** 117-28.

URBANOWICZ, R. A., LAMB, J. R., TODD, I., CORNE, J. M. & FAIRCLOUGH, L. C. (2009) Altered effector function of peripheral cytotoxic cells in COPD. *Respir Res,* 10**,** 53.

URBANOWICZ, R. A., LAMB, J. R., TODD, I., CORNE, J. M. & FAIRCLOUGH, L. C. (2010) Enhanced effector function of cytotoxic cells in the induced sputum of COPD patients. *Respir Res,* 11**,** 76.

VALES-GOMEZ, M., REYBURN, H. T., ERSKINE, R. A., LOPEZ-BOTET, M. & STROMINGER, J. L. (1999) Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2-A and the activating receptor CD94/NKG2-C to HLA-E. *Embo J,* 18**,** 4250-60.

VALIANTE, N. M., UHRBERG, M., SHILLING, H. G., LIENERT-WEIDENBACH, K., ARNETT, K. L., D'ANDREA, A., PHILLIPS, J. H., LANIER, L. L. & PARHAM, P. (1997) Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity,* 7**,** 739-51.

VAN DE GRIEND, R. J., BOLHUIS, R. L., STOTER, G. & ROOZEMOND, R. C. (1987) Regulation of cytolytic activity in CD3- and CD3+ killer cell clones by monoclonal antibodies (anti-CD16, anti-CD2, anti-CD3) depends on subclass specificity of target cell IgG-FcR. *J Immunol,* 138**,** 3137-44.

VAN DEN HEUVEL, M. M., TENSEN, C. P., VAN AS, J. H., VAN DEN BERG, T. K., FLUITSMA, D. M., DIJKSTRA, C. D., DOPP, E. A., DROSTE, A., VAN GAALEN, F. A., SORG, C., HOGGER, P. & BEELEN, R. H. (1999) Regulation of CD 163 on human macrophages: cross-linking of CD163 induces signaling and activation. *J Leukoc Biol,* 66**,** 858-66.

VAN GISBERGEN, K. P., SANCHEZ-HERNANDEZ, M., GEIJTENBEEK, T. B. & VAN KOOYK, Y. (2005) Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN. *J Exp Med,* 201**,** 1281-92.

VANDIVIER, R. W., FADOK, V. A., HOFFMANN, P. R., BRATTON, D. L., PENVARI, C., BROWN, K. K., BRAIN, J. D., ACCURSO, F. J. & HENSON, P. M. (2002) Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. *J Clin Invest,* 109**,** 661-70.

VASSALLO, R., TAMADA, K., LAU, J. S., KROENING, P. R. & CHEN, L. (2005) Cigarette smoke extract suppresses human dendritic cell function leading to preferential induction of Th-2 priming. *J Immunol,* 175**,** 2684-91.

VERMA, R. P. & HANSCH, C. (2007) Matrix metalloproteinases (MMPs): chemical-biological functions and (Q)SARs. *Bioorg Med Chem,* 15**,** 2223-68.

VERMAELEN, K. & PAUWELS, R. (2005) Pulmonary dendritic cells. *Am J Respir Crit Care Med,* 172**,** 530-51.

VERNERIS, M. R., KARAMI, M., BAKER, J., JAYASWAL, A. & NEGRIN, R. S. (2004) Role of NKG2D signaling in the cytotoxicity of activated and expanded CD8+ T cells. *Blood,* 103**,** 3065-72.

VERNOOY, J. H., KUCUKAYCAN, M., JACOBS, J. A., CHAVANNES, N. H., BUURMAN, W. A., DENTENER, M. A. & WOUTERS, E. F. (2002) Local and systemic inflammation in patients with chronic obstructive pulmonary disease: soluble tumor necrosis factor receptors are increased in sputum. *Am J Respir Crit Care Med,* 166**,** 1218-24.

VERNOOY, J. H., LINDEMAN, J. H., JACOBS, J. A., HANEMAAIJER, R. & WOUTERS, E. F. (2004) Increased activity of matrix metalloproteinase-8 and matrix metalloproteinase-9 in induced sputum from patients with COPD. *Chest,* 126**,** 1802-10.

VERNOOY, J. H., MOLLER, G. M., VAN SUYLEN, R. J., VAN SPIJK, M. P., CLOOTS, R. H., HOET, P. H., PENNINGS, H. J. & WOUTERS, E. F. (2007) Increased granzyme A expression in type II pneumocytes of patients with severe chronic obstructive pulmonary disease. *Am J Respir Crit Care Med,* 175**,** 464-72.

VERRECK, F. A., DE BOER, T., LANGENBERG, D. M., VAN DER ZANDEN, L. & OTTENHOFF, T. H. (2006) Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation. *J Leukoc Biol,* 79**,** 285-93.

VIEGI, G. & DI PEDE, C. (2002) Chronic obstructive lung diseases and occupational exposure. *Curr Opin Allergy Clin Immunol,* 2**,** 115-21.

VIJAYANAND, P., SEUMOIS, G., PICKARD, C., POWELL, R. M., ANGCO, G., SAMMUT, D., GADOLA, S. D., FRIEDMANN, P. S. & DJUKANOVIC, R. (2007) Invariant natural killer T cells in asthma and chronic obstructive pulmonary disease. *N Engl J Med,* 356**,** 1410-22.

VOYNOW, J. A., YOUNG, L. R., WANG, Y., HORGER, T., ROSE, M. C. & FISCHER, B. M. (1999) Neutrophil elastase increases MUC5AC mRNA and protein expression in respiratory epithelial cells. *Am J Physiol,* 276**,** L835-43.

WAGENA, E. J., ARRINDELL, W. A., WOUTERS, E. F. & VAN SCHAYCK, C. P. (2005) Are patients with COPD psychologically distressed? *Eur Respir J,* 26**,** 242-8.

WAGTMANN, N., BIASSONI, R., CANTONI, C., VERDIANI, S., MALNATI, M. S., VITALE, M., BOTTINO, C., MORETTA, L., MORETTA, A. & LONG, E. O. (1995) Molecular clones of the p58 NK cell receptor reveal immunoglobulin-related molecules with diversity in both the extra- and intracellular domains. *Immunity,* 2**,** 439-49.

WALDMANN, A. T. (2006) The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. *Nature Reviews,* 6**,** 595-601.

WALDMANN, T. (2002) The contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for the immunotherapy of rheumatological diseases. *Arthritis Res,* 4 Suppl 3**,** S161-7.

WALDMANN, T., TAGAYA, Y. & BAMFORD, R. (1998) Interleukin-2, interleukin-15, and their receptors. *Int Rev Immunol,* 16**,** 205-26.

WALDMANN, T. A. (1991) The interleukin-2 receptor. *J Biol Chem,* 266**,** 2681-4.

WALDMANN, T. A., DUBOIS, S. & TAGAYA, Y. (2001) Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. *Immunity,* 14**,** 105-10.

WALDMANN, T. A. & TAGAYA, Y. (1999) The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell

differentiation and host response to intracellular pathogens. *Annu Rev Immunol,* 17**,** 19-49.

WALLACE, A. M., SANDFORD, A. J., ENGLISH, J. C., BURKETT, K. M., LI, H., FINLEY, R. J., MULLER, N. L., COXSON, H. O., PARE, P. D. & ABBOUD, R. T. (2008) Matrix metalloproteinase expression by human alveolar macrophages in relation to emphysema. *Copd,* 5**,** 13-23.

WALLACE, W. A., GILLOOLY, M. & LAMB, D. (1992) Intra-alveolar macrophage numbers in current smokers and non-smokers: a morphometric study of tissue sections. *Thorax,* 47**,** 437-40.

WALSH, E. R., STOKES, K. & AUGUST, A. (2010) The role of eosinophils in allergic airway inflammation. *Discov Med,* 9**,** 357-62.

WALZ, A. (1992) Generation and properties of neutrophil-activating peptide 2. *Cytokines,* 4**,** 77-95.

WAN, Y. Y. (2010) Multi-tasking of helper T cells. *Immunology,* 130**,** 166-71.

WANG, J., URBANOWICZ, R. A., TIGHE, P. J., TODD, I., CORNE, J. M. & FAIRCLOUGH, L. C. (2013) Differential activation of killer cells in the circulation and the lung: a study of current smoking status and chronic obstructive pulmonary disease (COPD). *PLoS One,* 8**,** e58556.

WATANABE, T., JONO, H., HAN, J., LIM, D. J. & LI, J. D. (2004) Synergistic activation of NF-kappaB by nontypeable Haemophilus influenzae and tumor necrosis factor alpha. *Proc Natl Acad Sci U S A,* 101**,** 3563-8.

WATFORD, W. T., MORIGUCHI, M., MORINOBU, A. & O'SHEA, J. J. (2003) The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Rev,* 14**,** 361-8.

WATKINS, L. R., HANSEN, M. K., NGUYEN, K. T., LEE, J. E. & MAIER, S. F. (1999) Dynamic regulation of the proinflammatory cytokine, interleukin-1beta: molecular biology for non-molecular biologists. *Life Sci,* 65**,** 449-81.

WAUGH, D. J. & WILSON, C. (2008) The interleukin-8 pathway in cancer. *Clin Cancer Res,* 14**,** 6735-41.

WEDZICHA, J. A., SEEMUNGAL, T. A., MACCALLUM, P. K., PAUL, E. A., DONALDSON, G. C., BHOWMIK, A., JEFFRIES, D. J. & MEADE, T. W. (2000) Acute exacerbations of chronic obstructive pulmonary disease are accompanied by elevations of plasma fibrinogen and serum IL-6 levels. *Thromb Haemost,* 84**,** 210-5.

WEISS, S. J. (1989) Tissue destruction by neutrophils. *N Engl J Med,* 320**,** 365-76.

WEN, Y., REID, D. W., ZHANG, D., WARD, C., WOOD-BAKER, R. & WALTERS, E. H. (2010) Assessment of airway inflammation using sputum, BAL, and endobronchial biopsies in current and ex-smokers with established COPD. *Int J Chron Obstruct Pulmon Dis,* 5**,** 327-34.

WHITE, L., KRISHNAN, S., STRBO, N., LIU, H., KOLBER, M. A., LICHTENHELD, M. G., PAHWA, R. N. & PAHWA, S. (2007) Differential effects of IL-21 and IL-15 on perforin expression, lysosomal degranulation, and proliferation in CD8 T cells of patients with human immunodeficiency virus-1 (HIV). *Blood,* 109**,** 3873-80.

WILLIAMS, N. S., KLEM, J., PUZANOV, I. J., SIVAKUMAR, P. V., BENNETT, M. & KUMAR, V. (1999) Differentiation of NK1.1+,

Ly49+ NK cells from flt3+ multipotent marrow progenitor cells. *J Immunol,* 163**,** 2648-56.

WINTER, C. C., GUMPERZ, J. E., PARHAM, P., LONG, E. O. & WAGTMANN, N. (1998) Direct binding and functional transfer of NK cell inhibitory receptors reveal novel patterns of HLA-C allotype recognition. *J Immunol,* 161**,** 571-7.

WITTAMER, V., BONDUE, B., GUILLABERT, A., VASSART, G., PARMENTIER, M. & COMMUNI, D. (2005) Neutrophil-mediated maturation of chemerin: a link between innate and adaptive immunity. *J Immunol,* 175**,** 487-93.

WOOD, A. M. & STOCKLEY, R. A. (2006) The genetics of chronic obstructive pulmonary disease. *Respir Res,* 7**,** 130.

WOOLHOUSE, I. S., BAYLEY, D. L., LALOR, P., ADAMS, D. H. & STOCKLEY, R. A. (2005) Endothelial interactions of neutrophils under flow in chronic obstructive pulmonary disease. *Eur Respir J,* 25**,** 612-7.

WOOLHOUSE, I. S., BAYLEY, D. L. & STOCKLEY, R. A. (2002) Sputum chemotactic activity in chronic obstructive pulmonary disease: effect of alpha(1)-antitrypsin deficiency and the role of leukotriene B(4) and interleukin 8. *Thorax,* 57**,** 709-14.

WU, J., SONG, Y., BAKKER, A. B., BAUER, S., SPIES, T., LANIER, L. L. & PHILLIPS, J. H. (1999) An activating immunoreceptor complex formed by NKG2D and DAP10. *Science,* 285**,** 730-2.

WWW.GOLDCOPD.ORG. (2011) Global Initiative for chronic obstructive lung disease: Global strategy for the diagnosis, management, and prevention of COPD.

WYATT, T. A., HEIRES, A. J., SANDERSON, S. D. & FLOREANI, A. A. (1999) Protein kinase C activation is required for cigarette smoke-enhanced C5a-mediated release of interleukin-8 in human bronchial epithelial cells. *Am J Respir Cell Mol Biol,* 21**,** 283-8.

XU, X., WEISS, S. T., RIJCKEN, B. & SCHOUTEN, J. P. (1994) Smoking, changes in smoking habits, and rate of decline in FEV1: new insight into gender differences. *Eur Respir J,* 7**,** 1056-61.

YAMAMOTO, C., YONEDA, T., YOSHIKAWA, M., FU, A., TOKUYAMA, T., TSUKAGUCHI, K. & NARITA, N. (1997) Airway inflammation in COPD assessed by sputum levels of interleukin-8. *Chest,* 112**,** 505-10.

YANG, X., STENNICKE, H. R., WANG, B., GREEN, D. R., JANICKE, R. U., SRINIVASAN, A., SETH, P., SALVESEN, G. S. & FROELICH, C. J. (1998) Granzyme B mimics apical caspases. Description of a unified pathway for trans-activation of executioner caspase-3 and -7. *J Biol Chem,* 273**,** 34278-83.

YASUDA, N., GOTOH, K., MINATOGUCHI, S., ASANO, K., NISHIGAKI, K., NOMURA, M., OHNO, A., WATANABE, M., SANO, H., KUMADA, H., SAWA, T. & FUJIWARA, H. (1998) An increase of soluble Fas, an inhibitor of apoptosis, associated with progression of COPD. *Respir Med,* 92**,** 993-9.

YEN, J. H., MOORE, B. E., NAKAJIMA, T., SCHOLL, D., SCHAID, D. J., WEYAND, C. M. & GORONZY, J. J. (2001) Major histocompatibility complex class I-recognizing receptors are disease risk genes in rheumatoid arthritis. *J Exp Med,* 193**,** 1159-67.

YING, S., O'CONNOR, B., RATOFF, J., MENG, Q., FANG, C., COUSINS, D., ZHANG, G., GU, S., GAO, Z., SHAMJI, B., EDWARDS, M. J., LEE, T. H. & CORRIGAN, C. J. (2008) Expression and cellular provenance of thymic stromal lymphopoietin and chemokines in patients with severe asthma and chronic obstructive pulmonary disease. *J Immunol,* 181**,** 2790-8.

YOKOHORI, N., AOSHIBA, K. & NAGAI, A. (2004) Increased levels of cell death and proliferation in alveolar wall cells in patients with pulmonary emphysema. *Chest,* 125**,** 626-32.

YOKOMIZO, T., KATO, K., TERAWAKI, K., IZUMI, T. & SHIMIZU, T. (2000) A second leukotriene B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders. *J Exp Med,* 192**,** 421-32.

YOSHIMURA, A. & MUTO, G. (2011) TGF-beta function in immune suppression. *Curr Top Microbiol Immunol,* 350**,** 127-47.

YOSHIOKA, A., BETSUYAKU, T., NISHIMURA, M., MIYAMOTO, K., KONDO, T. & KAWAKAMI, Y. (1995) Excessive neutrophil elastase in bronchoalveolar lavage fluid in subclinical emphysema. *Am J Respir Crit Care Med,* 152**,** 2127-32.

YU, H., FEHNIGER, T. A., FUCHSHUBER, P., THIEL, K. S., VIVIER, E., CARSON, W. E. & CALIGIURI, M. A. (1998) Flt3 ligand promotes the generation of a distinct CD34(+) human natural killer cell progenitor that responds to interleukin-15. *Blood,* 92**,** 3647-57.

YU, K. O., IM, J. S., MOLANO, A., DUTRONC, Y., ILLARIONOV, P. A., FORESTIER, C., FUJIWARA, N., ARIAS, I., MIYAKE, S., YAMAMURA, T., CHANG, Y. T., BESRA, G. S. & PORCELLI, S. A. (2005) Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. *Proc Natl Acad Sci U S A,* 102**,** 3383-8.

ZAGURY, D. (1982) Direct analysis of individual killer T cells: susceptibility of target cells to lysis and secretion of hydrolytic enzymes by CTL. *Adv Exp Med Biol,* 146**,** 149-69.

ZDRENGHEA, M. T., TELCIAN, A. G., LAZA-STANCA, V., BELLETTATO, C. M., EDWARDS, M. R., NIKONOVA, A., KHAITOV, M. R., AZIMI, N., GROH, V., MALLIA, P., JOHNSTON, S. L. & STANCIU, L. A. (2012) RSV infection modulates IL-15 production and MICA levels in respiratory epithelial cells. *Eur Respir J,* 39**,** 712-20.

ZHANG, D., SHANKAR, P., XU, Z., HARNISCH, B., CHEN, G., LANGE, C., LEE, S. J., VALDEZ, H., LEDERMAN, M. M. & LIEBERMAN, J. (2003) Most antiviral CD8 T cells during chronic viral infection do not express high levels of perforin and are not directly cytotoxic. *Blood,* 101**,** 226-35.

ZHANG, J., HE, J., XIA, J., CHEN, Z. & CHEN, X. (2012) Delayed apoptosis by neutrophils from COPD patients is associated with altered Bak, Bcl-xl, and Mcl-1 mRNA expression. *Diagn Pathol,* 7**,** 65.

ZHANG, K., SHAN, L., RAHMAN, M. S., UNRUH, H., HALAYKO, A. J. & GOUNNI, A. S. (2007) Constitutive and inducible thymic stromal lymphopoietin expression in human airway smooth muscle cells: role

in chronic obstructive pulmonary disease. *Am J Physiol Lung Cell Mol Physiol,* 293**,** L375-82.

ZHANG, X., SUN, S., HWANG, I., TOUGH, D. F. & SPRENT, J. (1998) Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. *Immunity,* 8**,** 591-9.

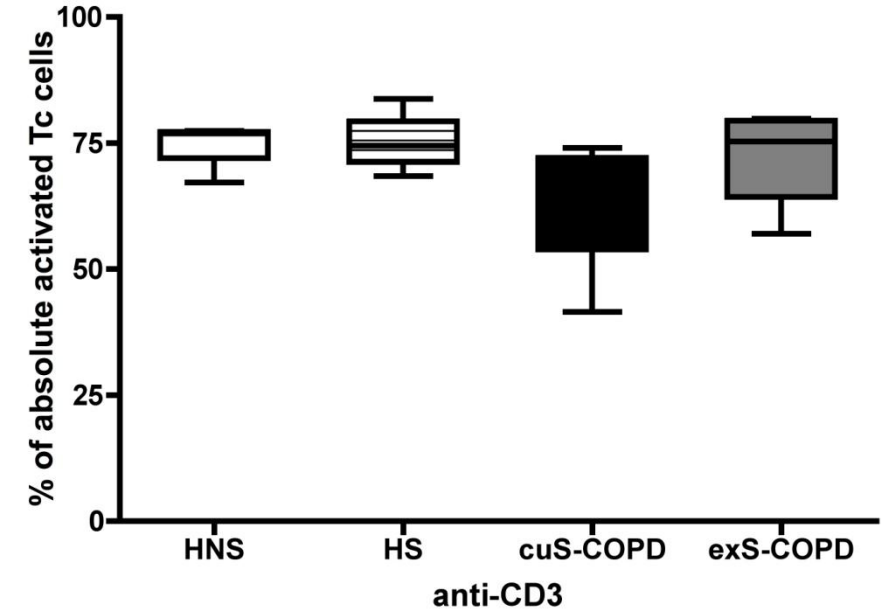
ZHOU, D., MATTNER, J., CANTU, C., 3RD, SCHRANTZ, N., YIN, N., GAO, Y., SAGIV, Y., HUDSPETH, K., WU, Y. P., YAMASHITA, T., TENEBERG, S., WANG, D., PROIA, R. L., LEVERY, S. B., SAVAGE, P. B., TEYTON, L. & BENDELAC, A. (2004) Lysosomal glycosphingolipid recognition by NKT cells. *Science,* 306**,** 1786-9.

ZHU, J., QIU, Y. S., MAJUMDAR, S., GAMBLE, E., MATIN, D., TURATO, G., FABBRI, L. M., BARNES, N., SAETTA, M. & JEFFERY, P. K. (2001) Exacerbations of Bronchitis: bronchial eosinophilia and gene expression for interleukin-4, interleukin-5, and eosinophil chemoattractants. *Am J Respir Crit Care Med,* 164**,** 109-16.

ZIELINSKI, J. & KUCA, P. (2004) [Alpha-1 antitrypsin deficiency: a cause of COPD not sufficiently recognized in Poland]. *Pneumonol Alergol Pol,* 72**,** 364-6.

# Appendix

**A**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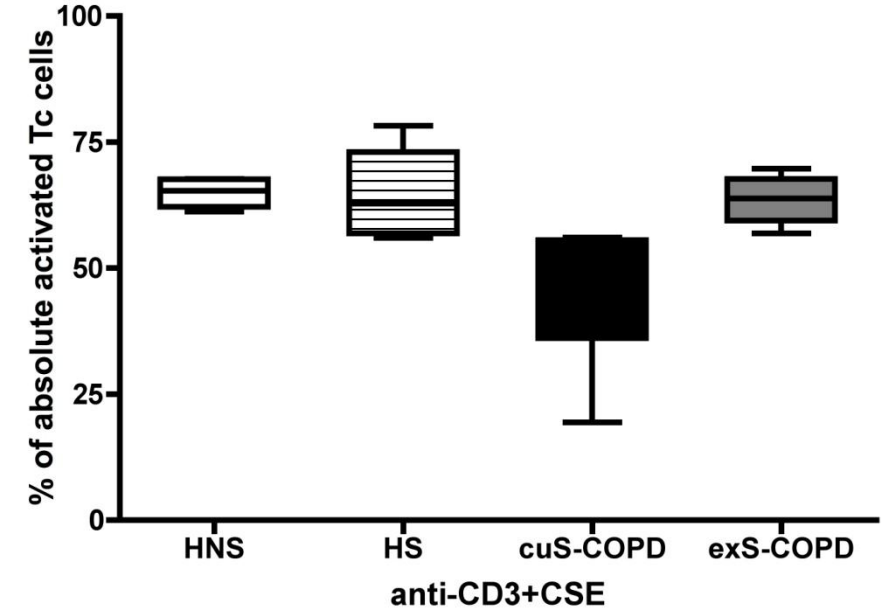
HNS
HS
cuS-COPD
exS-COPD
anti-CD3

**B**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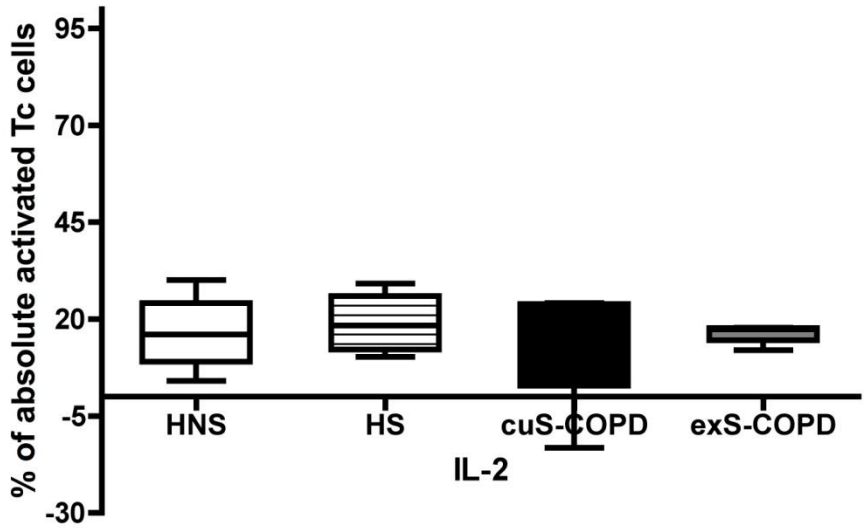
exS-COPD
anti-CD3+CSE

**C**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HS
cuS-COPD
exS-COPD
IL-2

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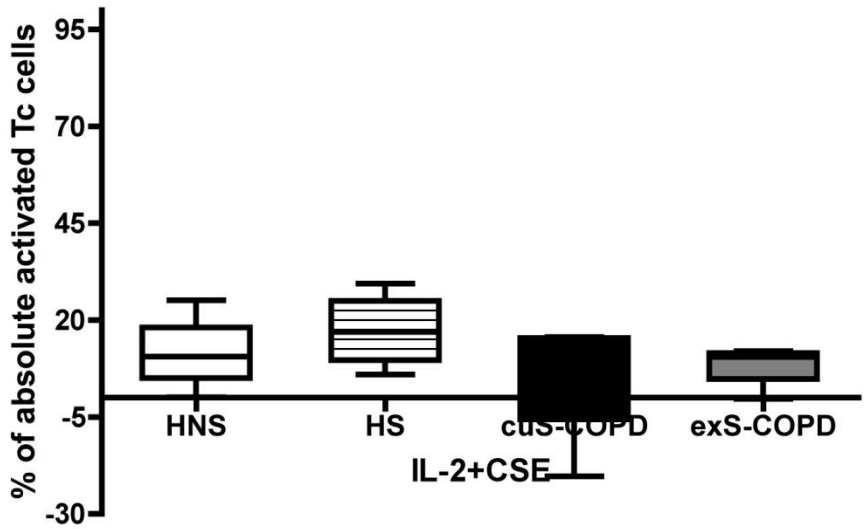
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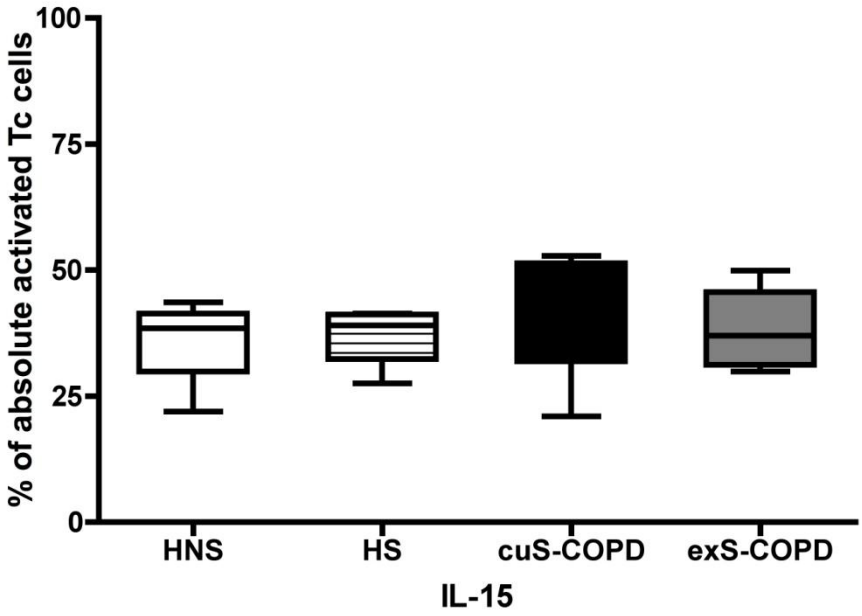
cuS-COPD
exS-COPD
IL-2+CSE

**E**

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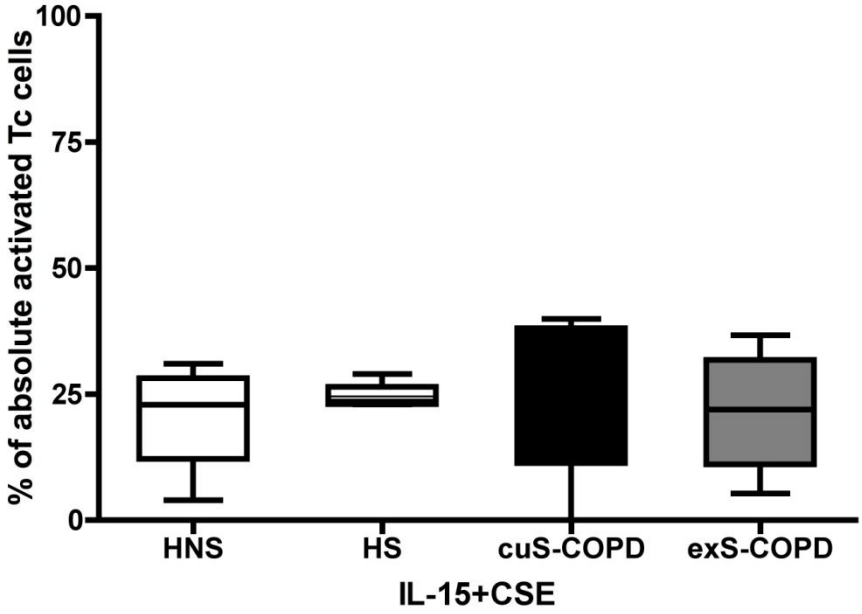
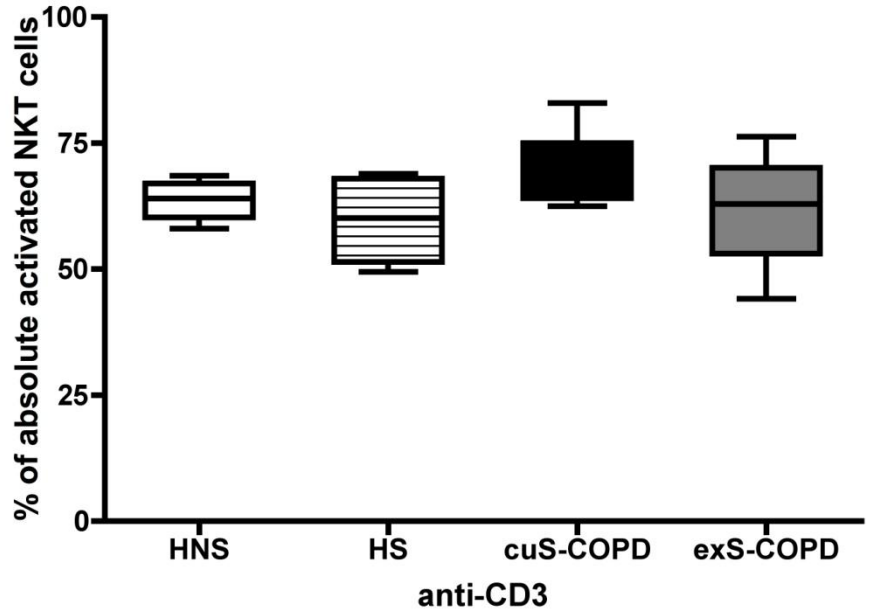



**Figure 1. Comparison of absolute activation levels of $CD8^+$ T cells upon different stimulation in four groups including healthy non-smokers (HNS, n=4), current healthy smokers (HS, n=4), current smokers with COPD (cuS-COPD, n=4) and ex-smokers with COPD (exS-COPD, n=4).** PBMCs were collected and incubated with different reagents for 48 hours.

**A**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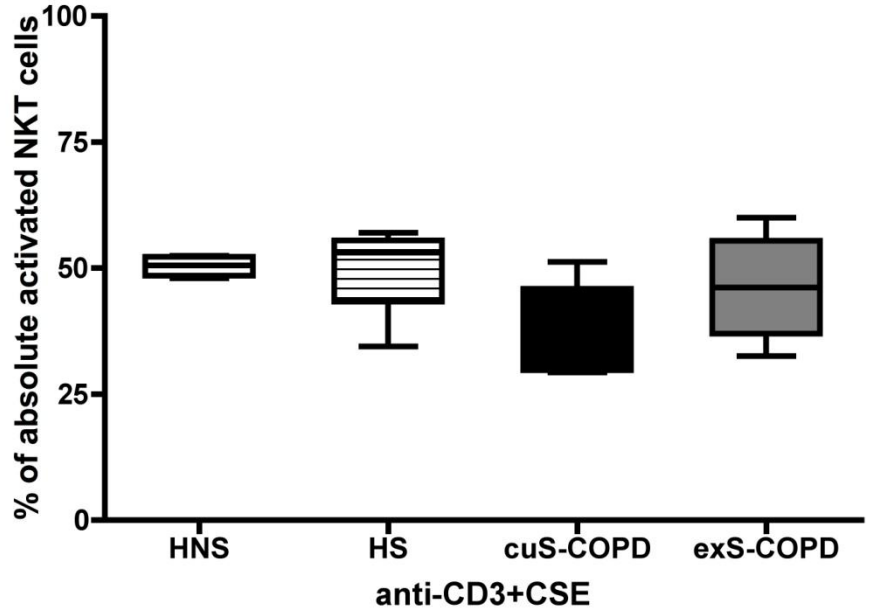
75
50
25
0
HNS
HS
cuS-COPD
exS-COPD
anti-CD3

**B**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HNS
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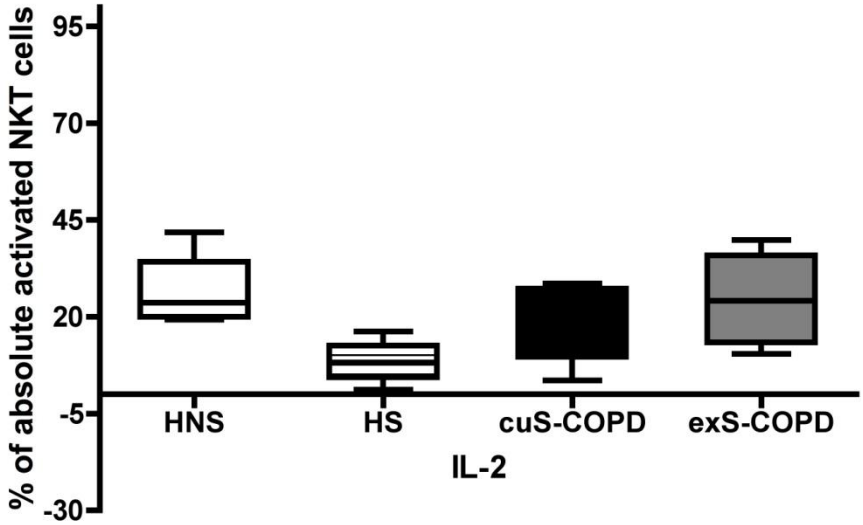
cuS-COPD
exS-COPD
anti-CD3+CSE

**C**


% of absolute activated NKT cells
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70
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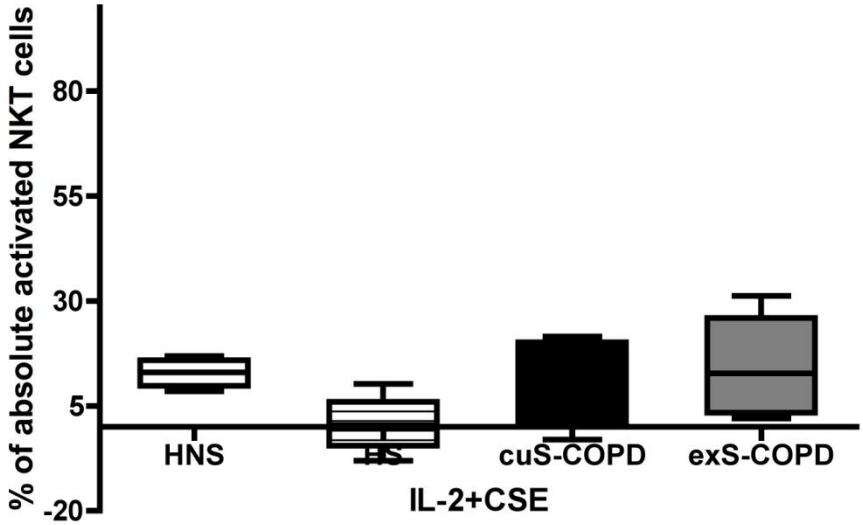
HNS
HS
cuS-COPD
exS-COPD
IL-2

**D**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IL-2+CSE

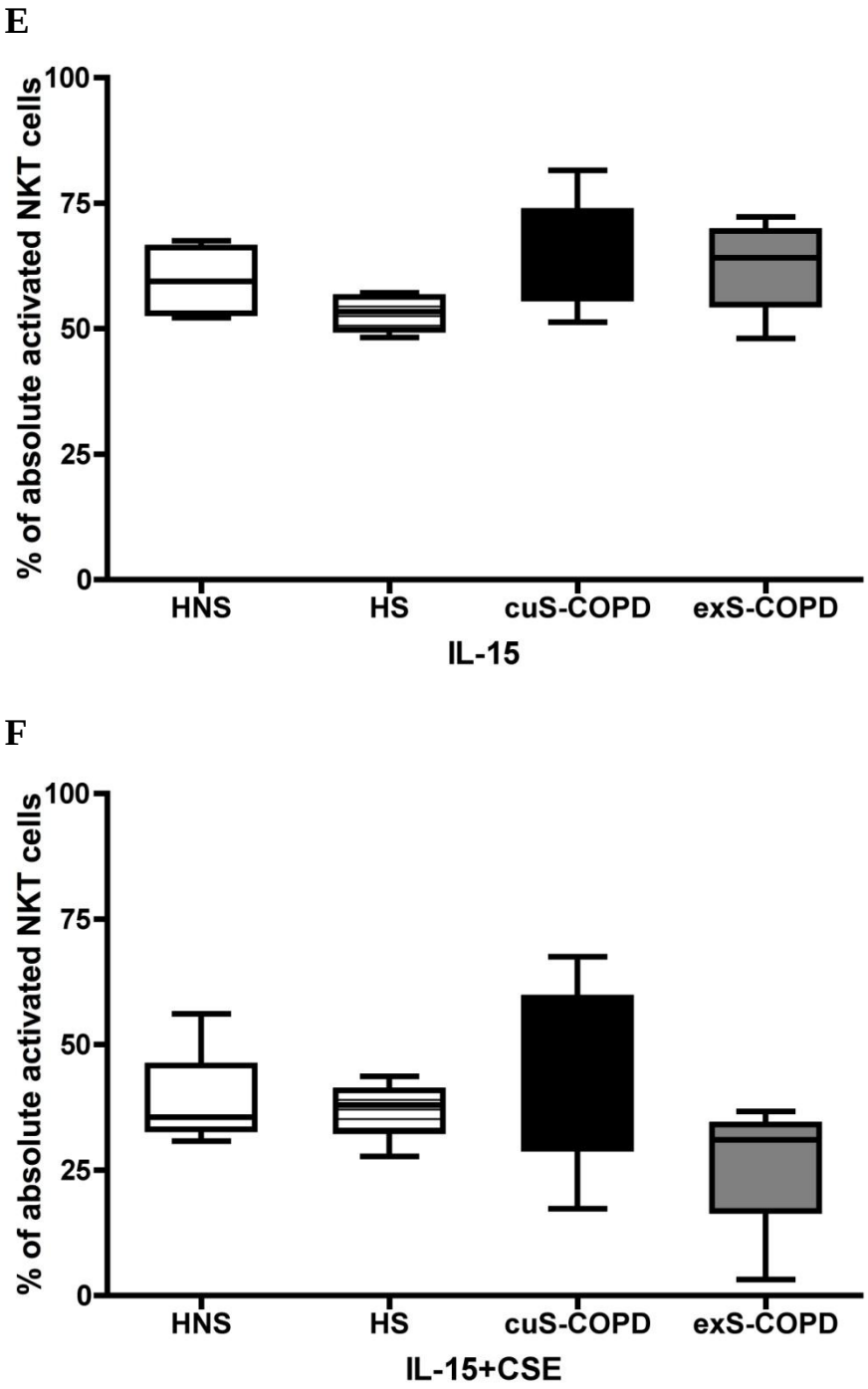
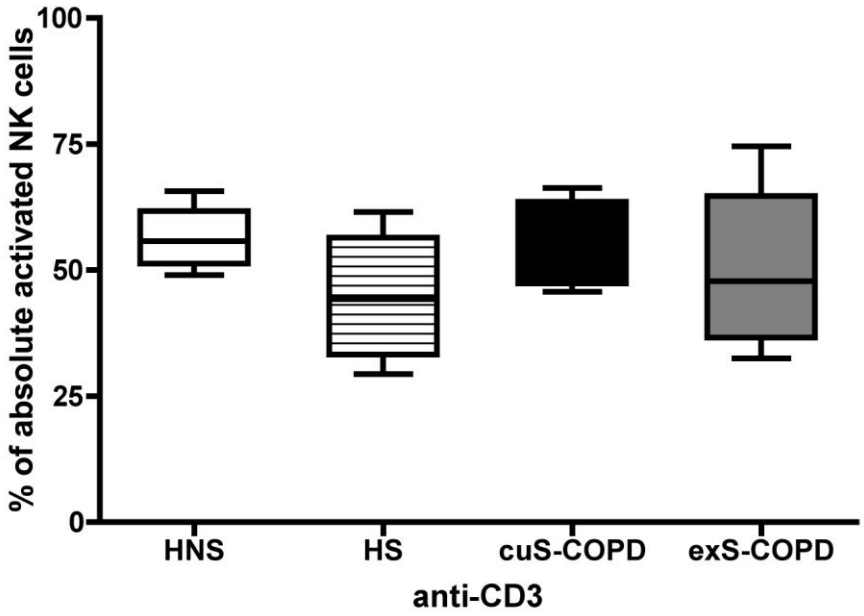



**Figure 2. Comparison of absolute activation levels of NKT-like cells upon different stimulation in four groups including healthy non-smokers (HNS, n=4), current healthy smokers (HS, n=4), current smokers with COPD (cuS-COPD, n=4) and ex-smokers with COPD (exS-COPD, n=4).** PBMCs were collected and incubated with different reagents for 48 hours.

**A**


% of absolute activated NK cells
100
75
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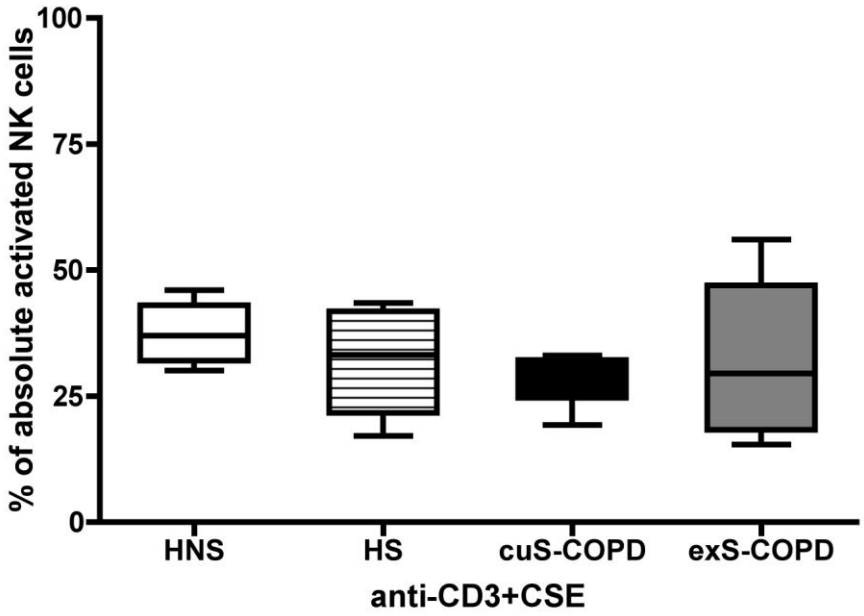
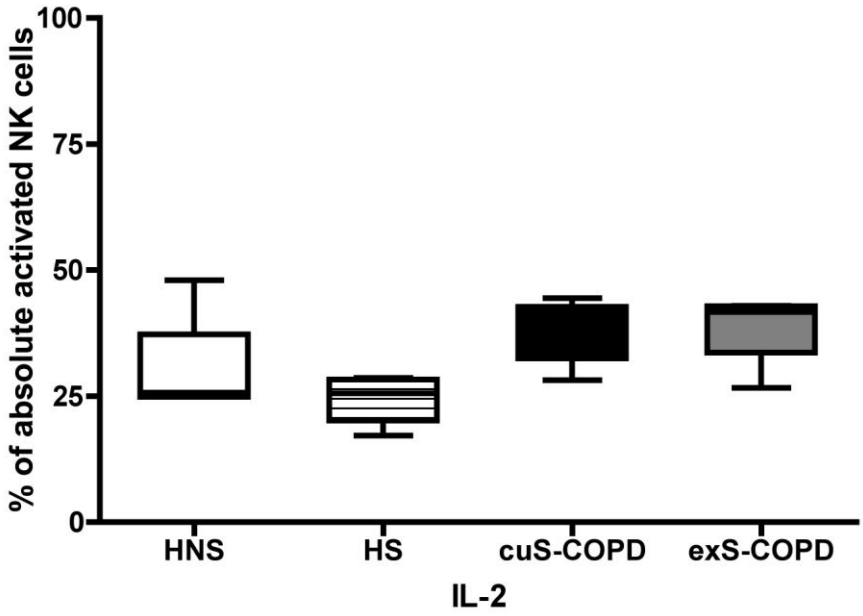
HNS
HS
cuS-COPD
exS-COPD
anti-CD3

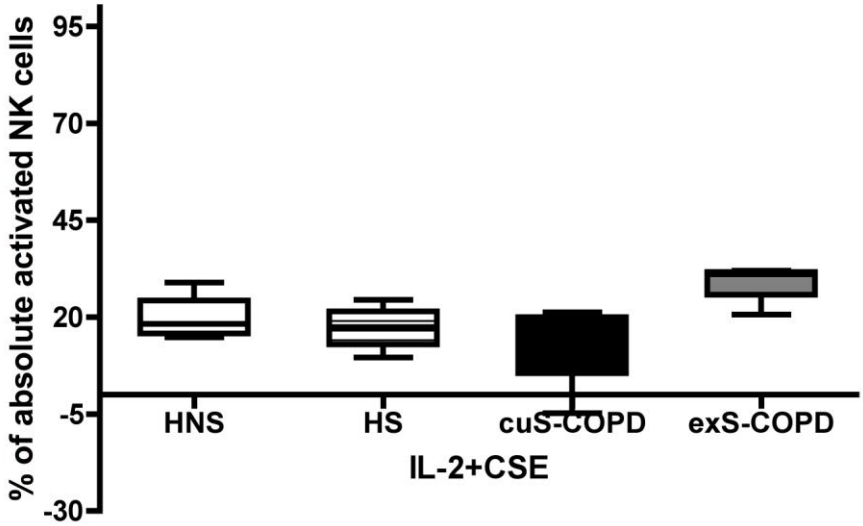
**B**


% of absolute activated NK cells
100
75
50
25
0
HNS
HS
cuS-COPD
exS-COPD
anti-CD3+CSE

**C**


% of absolute activated NK cells
100
75
50
25
0
HNS
HS
cuS-COPD
exS-COPD
IL-2

**D**


% of absolute activated NK cells
95
70
45
20
-5
-30
HNS
HS
cuS-COPD
exS-COPD
IL-2+CSE

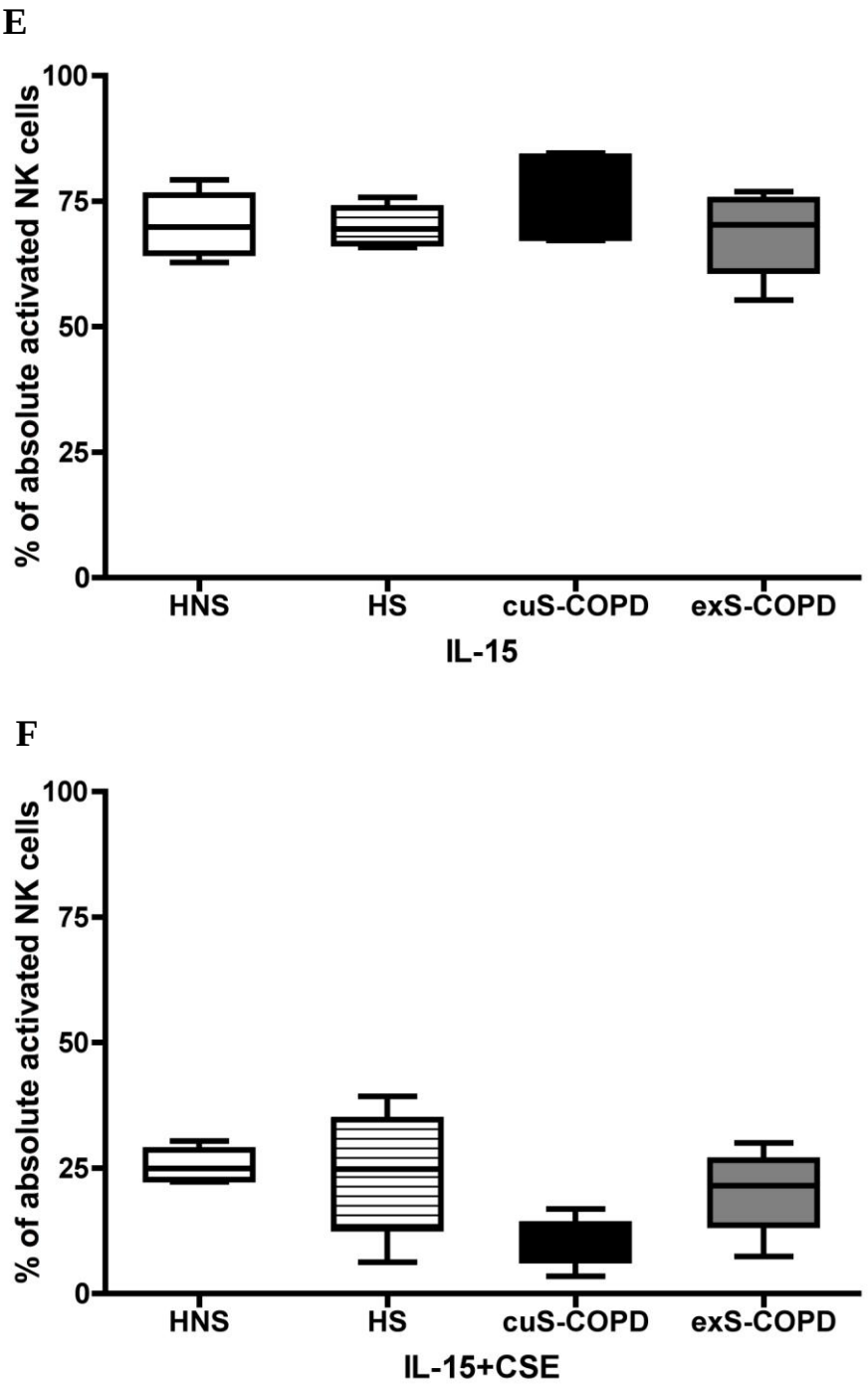



**Figure 3. Comparison of absolute activation levels of NK cells upon different stimulation in four groups including healthy non-smokers (HNS, n=4), current healthy smokers (HS, n=4), current smokers with COPD (cuS-COPD, n=4) and ex-smokers with COPD (exS-COPD, n=4).** PBMCs were collected and incubated with different reagents for 48 hours.